Funktionelle Analyse der Protein-Tyrosinphosphatase DEP-1 in Kolon-Epithelzellen

DISSERTATION

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ZUSAMMENFASSUNG

Die Tyrosinphosphorylierung ist ein wichtiger Signalmechanismus in eukaryotischen Zellen. Sie wird durch die gegensätzliche Aktivität von Protein-Tyrosinkinasen (PTK) und Protein-Tyrosinphosphatasen (PTP) gesteuert. Im Rahmen dieser Arbeit wurde die funktionelle Rolle der PTP mit dem Namen „density-enhanced phosphatase-1“ (DEP-1) in Kolonepithelzellen untersucht. DEP-1 (auch als PTPRJ, PTPη, oder CD 148 bezeichnet) ist eine ubiquitär exprimierte Transmembran-PTP. Sie zeichnet sich strukturell durch 8 Fibronektin-ähnliche Repeats in der extrazellulären Domäne und eine einzelne katalytische Domäne auf der zytoplasmatischen Seite der Membran aus. DEP-1 wurde als Tumorsuppressor für verschiedene epitheliale Tumoren vorgeschlagen, darunter Mammakarzinom, Schilddrüsen-Karzinom, Gliom und Pankreas-Karzinom. Der in Mäusen identifizierte „colon cancer susceptibility locus- 1“ (*Scc 1*) enthält das DEP-1-kodierende Gen *Ptprj*. Die physiologische Rolle von DEP-1 in Kolonepithelzellen ist jedoch bisher unbekannt.

Die Analyse der endogenen Expression des DEP-1 Proteins in einer Serie von Kolonepithel-Zelllinien zeigte, dass die Expressionsspiegel sehr unterschiedlich waren. Hohe Spiegel wurden in der Adenom-Zelllinie LT97 und der Karzinom-Zelllinien HT29 gefunden, während die Karzinom-Zelllinie SW480 keine endogene Proteinexpression aufwies. SW480 Zellen wurde deshalb ausgewählt, um humane aktive DEP-1 (Wildtyp, WT) und die katalytisch inaktive Version mit der Mutation C1239S konditionell zu re-exprimieren. Die Expression von DEP-1 WT hemmte die Proliferation der Zellen, wohingegen die katalytisch inaktive Mutante keinen Einfluss darauf hatte. Die Unterdrückung der Expression von DEP-1 in HT29 Zellen förderte die Proliferation dieser Zellen, was die anti-proliferative Aktivität von DEP-1 bestätigte. Wenn SW480 Zellen mit Hepatozyten-Wachstumsfaktor (HGF) stimuliert wurden, führte die Re-Expression von DEP-1 WT zu einer starken Reduktion der Phosphorylierung des HGF-Rezeptors und der durch HGF aktivierten Kinase Erk. Wie bei den genannten Proliferations-Assays, hängt dieser Effekt von der Phosphatase-Aktivität von DEP-1 ab und wurde in Zellen, welche die katalytisch inaktive DEP-1 C1239S Variante

exprimierten nicht beaobachtet. Die negative Regulation dieses Signalweges könnte teilweise an der anti-proliferativen Aktivität von DEP-1 beteiligt sein.

Re-Expression von DEP-1 hemmte auch die Zellmigration und induzierte starke Änderungen des Zytoskeletts. DEP-1 WT exprimierende Zellen waren abgeflacht und zeigten eine andere Verteilung des filamentösen Aktins, typischerweise charakterisiert durch kortikale Aktinbündel unterhalb der Zellperipherie. Die Zytoskelett-Veränderungen kamen auch in einer veränderten Verteilung von Paxillin, Phospho-Paxillin und Vinculin in der Zellperipherie zum Ausdruck. Die DEP-1 Expression induzierte eine erhöhte Phosphorylierung von Paxillin und der Fokalen Adhäsions-Kinase (FAK).

Die Re-Expression von DEP-1 machte die Zellen sensitiver gegen die Induktion von Apoptose, was sich auch in verringerten Spiegeln der phosphorylierten Akt-Kinase ausdrückte. Ein komplementierender Befund war die erhöhte Phosphorylierung von Akt in Zellen mit unterdrückter DEP-1 Expression. Diese Daten legen nahe, dass die Regulation der Phosphorylierung von Akt eine der physiologischen Funktionen von DEP-1 darstellt. Die relevanten Targets von DEP-1 „stromaufwärts“ von Akt sind noch nicht bekannt. Die Analyse der Effekte der DEP-1 Expression auf 42 verschiedene Rezeptortyrosinkinasen (RTK) in einem Antikörper-Array ergab keine wesentlichen Unterschiede in der Phosphorylierung dieser Kinasen. Dieser Befund legt nahe, dass die relevanten Targets von DEP-1 in den untersuchten Zellen nicht unter diesen Kinasen zu finden sind.

Eine Anzahl von Nahrungsinhaltsstoffen und ihren Metaboliten wirken wahrscheinlich protektiv bezüglich der Entwicklung von Kolonkarzinomen. Wenn Kolonepithelzellen mit solchen Nahrungskomponenten (Apfel-Polyphenolextrakt, Butyrat, Extrakt aus Grünem Tee) behandelt wurden, erhöhte sich die Expression des DEP-1 Proteins. Damit wurde erstmals die Induktion eines Tumorsuppressors durch protektive Nahrungsbestandteile nachgewiesen. Die Daten legen nahe, dass die Induktion von DEP-1 einen bisher unerkannten Mechanismus der Chemoprävention durch Nahrungsinhaltsstoffe darstellen könnte.

Functional Analysis of DEP-1 Protein Tyrosine Phosphatase in Colon Epithelial Cells

Thesis

For acquiring the academic degree
Doctor rerum naturalium

Presented to the Faculty of Biology and Pharmacy
Friedrich Schiller University, Jena

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from Musiri / India

SUMMARY

Tyrosine phosphorylation is an important signaling mechanism in eukaryotic cells. It is governed by the balanced action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Here we have analysed the functional role of a protein tyrosine phosphatase called Density Enhanced Phosphatase-1 (DEP-1) in colon epithelial cells. DEP-1 (also referred to as PTPRJ, Ptpη or CD 148) is a ubiquitously expressed transmembrane protein-tyrosine phosphatase. It has eight fibronectin repeats on the extracellular side and a single catalytical domain on the cytoplasmic side. Its expression is increased upon high cell density in a number of cell lines. It has been proposed as a tumor suppressor in several epithelial cancers such as mammary carcinoma, thyroid carcinoma, glioma and pancreatic carcinomas. Furthermore, the colon cancer susceptibility locus (*Scc 1*) in mice harbors the *Ptprj* gene but its physiological role in colon cells is not known.

Analysis of the endogenous level of DEP-1 expression in a set of colon epithelial cell lines revealed its expression to be quite heterogeneous; higher levels were found in the adenoma cell line LT 97 and the carcinoma cell line HT 29 whereas there was no detectable expression in the cell line SW 480. We conditionally re-expressed either the wild type human DEP-1 or the catalytically inactive C1239S mutant into the DEP-1-negative cell line SW 480. The re-expression of DEP-1 WT impaired proliferation, whereas the catalytically inactive mutant had no effect on it. Moreover, suppression of DEP-1 in the HT 29 cell line, using shRNA (short hairpin RNA), enhanced the proliferation of these cells, confirming the anti-proliferative effect of DEP-1. When stimulated with hepatocyte growth factor (HGF), re-expression of DEP-1 leads to hypo-phosphorylation of HGFR and Erk. Similar to the proliferation assays mentioned above these effects depend on the PTP activity of DEP-1 as they were not observed with cells expressing the catalytically inactive DEP-1 C1239S variant. Negative regulation of this pathway may partially be responsible for the anti-proliferative activity of DEP-1.

Re-expression of DEP-1 also inhibited cell migration and induced severe cytoskeletal changes. DEP-1 WT expressing cells were flattened and exhibited a different distribution of filamentous actin, typically characterized by cortical actin bundles close to the cell periphery. These cytoskeletal changes were further emphasized by a differential pattern of distribution of paxillin, phospho-paxillin and vinculin in the cell periphery. DEP-1 expression induces hyper-phosphorylation of paxillin and Focal Adhesion Kinase (FAK).

The re-expression of DEP-1 sensitized the cells towards apoptosis which is also mirrored by the reduction of phospho-Akt levels. Complementing this finding, DEP-1 suppressed cell lines showed enhanced Akt phosphorylation. This emphasizes that the physiological regulation of Akt phosphorylation depends on DEP-1 expression. The relevant targets of DEP-1 “upstream” of Akt were yet unknown. Analysis of the effects of DEP-1 expression on 42 different receptor tyrosine kinases (RTKs) did not reveal any significant differences on their phosphorylation status. This suggests that these RTKs may not be the prime targets of DEP-1 in the cell lines investigated.

A number of food ingredients and their metabolites were proposed to be protective against colon cancer development. When colon epithelial cell lines were treated with such nutrient components (apple polyphenol extract, butyrate and green tea extract), the expression of DEP-1 was found to be upregulated. This is the first report where the effects of protective nutrients are attributed to the upregulation of a tumor suppressor. These data suggest that an upregulation of DEP-1 as a previously unrecognized aspect of chemoprevention by dietary compounds.

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1. Introduction

1.1. Cell Signaling

Cell signaling is a part of complex system of communication that governs basic cellular activities and coordinates cell's actions. A cell's ability to respond to its various extracellular stimuli results in a complex and highly organized series of intracellular events. In general, cellular signaling is initiated with the binding of the extracellular stimuli (growth factors, cytokines, hormones, neuro-transmitters etc.) to their cognate receptors. These receptors then amplify and transmit the signals intracellularly (usually involving protein phosphorylation) culminating with changes in the gene expression, thereby altering the biological properties of the cells such as proliferation, migration and differentiation. (Tischer and Bastiens 2003)

1.2. Protein Tyrosine Kinases

Protein kinases are important regulators of intracellular signal-transduction pathways mediating development and multicellular communication in metazoans (Blume-Jensen and Hunter 2001). The human genome has been found to contain 518 putative protein kinase genes. 478 of them fall under eukarytotic protein kinases (ePK) whereas 40 belong to atypical protein kinases (aPK) because they lack sequence similarity to the ePK domain. The kinase genome can also be classified into nine broad groups, 134 families and 201 sub-families including 106 pseudogenes (Manning, Whyte et al. 2002).

Phosphorylation of proteins on tyrosine residues acts as a reversible and specific switching mechanism which is controlled through the coordinated actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). There are 90 known protein tyrosine kinase genes (PTKs) in the human genome of which 58 encode for receptor protein tyrosine kinases (RTKs) that span the cell membrane (distributed into 20 subfamilies) and 32 encode for cytoplasmic, non-receptor PTKs (Blume-Jensen and Hunter 2001). RTKs are activated by an intermolecular mechanism whereas the

cytoplasmic tyrosine kinases are activated by both intra- and intermolecular mechanisms. (Weiss and Schlessinger 1998). RTKs are composed of an extracellular ligand binding domain which is usually glycosylated, a single transmembrane domain which spans the membrane and an intracellular kinase domain that contains key phospho-tyrosine residues. Ligand binding induces dimerization of these receptors resulting in autophosphorylation of their cytoplasmic domains (Heldin 1995). This activates the kinase, creating docking sites for other proteins which will then transmit the signals to the interior of the cell, usually the nucleus. Some of the important RTK family members which are extensively studied and have also been implicated in disease and cancer are Epidermal Growth Factor Receptor (EGFR), Hepatocyte Growth Factor Receptor (HGFR), Insulin Receptor (IR), and Platelet Derived Growth Factor Receptor (PDGFR). Among the cytoplasmic PTKs Src, Abl, Focal Adhesion Kinase (FAK) and Janus Kinase (JAK) were extensively studied. Fig. 1.1a and Fig. 1.1b refer to the broad classification of RTKs and cytoplasmic PTKs, respectively.

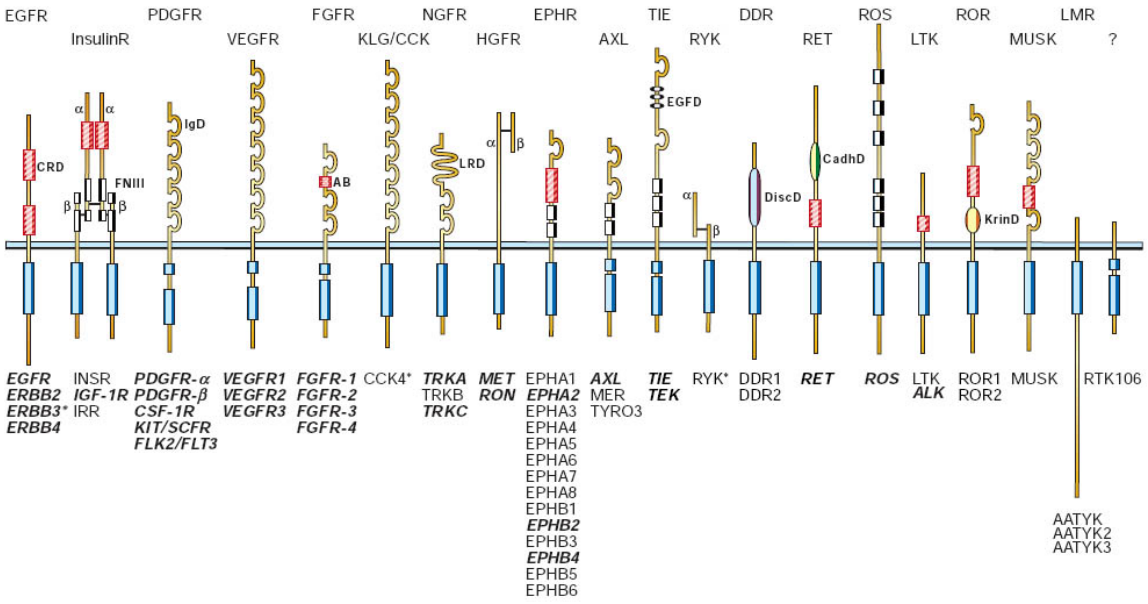


Fig. 1.1a The prototypic receptor for each family is indicated above the receptor, and the known members are listed below. The symbols alpha and beta denote distinct RTK subunits. RTK members in bold and italic type are implicated in human malignancies. An asterisk indicates that the member is devoid of intrinsic kinase activity [taken from (Blume-Jensen and Hunter 2001)].

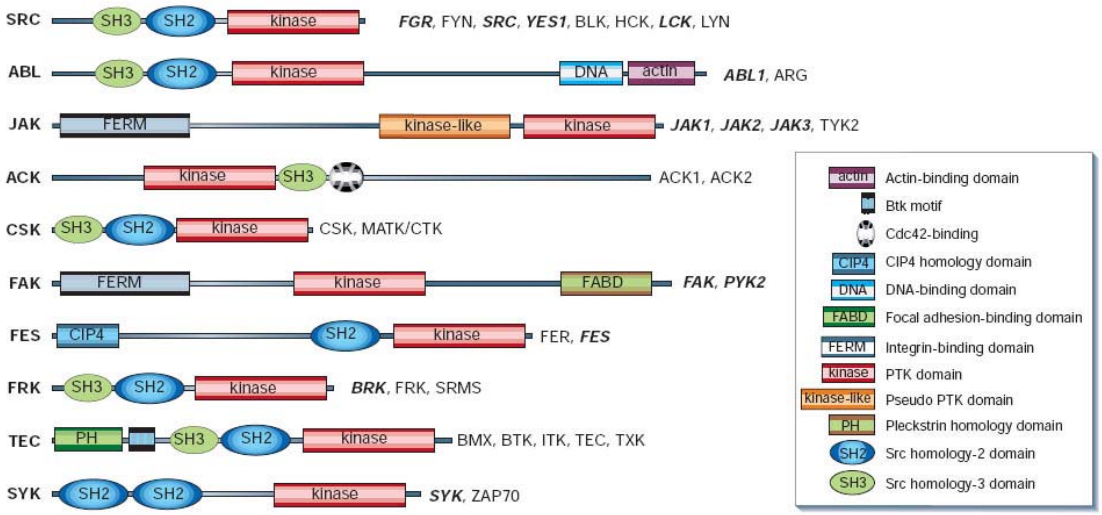


Fig. 1.1b The family members are indicated to the right and the family name to the left of each PTK. The PTK members in bold and italic type are implicated in human malignancies [taken from (Blume-Jensen and Hunter 2001)].

1.3. Serine / Threonine Phosphatases

Ser/Thr phosphatases represent a diverse family and are expressed in many cell types and different cellular compartments. They are regulated by several mechanisms and can be broadly classified into “PPP” and “PPM” families defined by amino acid sequence and 3-dimensional structure. The PPP family members are composed of catalytic and regulatory subunits, with each subunit being expressed in several isoforms by distinct genes/alternative splicing. On the contrary the PPM family members exist as a monomer devoid of regulatory subunits. The regulatory subunits of PPP family members have multiple functions including the control of catalytic activity, subcellular localization and substrate specificity of the phosphatase. Some of the members depend on specific bivalent cations for their activity. For instance, PP2B requires Ca^{2+} and calmodulin for full activity whereas PP2C needs either Mg^{2+} or Mn^{2+} (Gee and Mansuy 2005). The following table (Table 1.1) gives an overview of different Ser/Thr phosphatases.

Family	Subfamily	Genes	Catalytic subunits	Regulatory subunits	Subunit composition	Selected inhibitors
PPP	PP1 (PPP1)	3	α, β, γ1, γ2	>50	Heterodimer and higher order	Inhibitor-1, inhibitor-2, microcystin (MC), okadaic acid, calyculin A (Cal A)
	PP2A (PPP2)	2	α, β	PPP2R1–3 PPP2R5	Heterodimer and higher order	Fostriecin, okadaic acid, MC, Cal A, nodularin
	PP4 (PPP4) (PP2A-like)	1	1	IGBP1, PR4R1, PPP4R2, c-Re1	Monomer and higher order	Fostriecin, okadaic acid, MC, Cal A, nodularin
	PP6 (PPP6) (PP2A-like)	1	1	IGBP1	Monomer and higher order	not known
	PP2B (PPP3)	3	3	Calcineurin B, AKAPs, calmodulin	Heterodimer and higher order	not known
	PP5 (PPP5)	1	1	PP2A, A and B subunits	Monomer and higher order	Okadaic acid, MC, Cal A, nodularin
	PP7 (PPP7)	2	2	not known	Monomer	not known
PPM	PP2C	6	10	not known	Monomer	not known
FCP	FCP1	1	4	not known	Monomer	not known

Table 1.1: Classification of Ser/Thr phosphatases [adapted from (Gallego and Virshup 2005)].

1.4. Protein Tyrosine Phosphatases

Protein tyrosine phosphatases (PTPs) act by dephosphorylating tyrosine residues and hence antagonizing the actions of tyrosine kinases. They can antagonize both ligand-independent and ligand-activated RTK signaling (Ostman and Bohmer 2001). The first PTP to be purified was PTP 1B from human placenta in 1988 (Tonks, Diltz et al. 1988). Till date, 107 PTPs were found to be encoded by the human genome, and they fall into four major classes (Alonso, Sasin et al. 2004).

1.4.1. Classification of Protein Tyrosine Phosphatases

Based on the amino acid sequences of their catalytic domains, the PTPs can be grouped into four separate families (Fig. 1.2). Class I cysteine based PTPs constitute the largest family comprising 38 classical PTPs which are strictly tyrosine specific whereas the 61 dual specific phosphatases can dephosphorylate both phospho Ser/Thr and phospho Tyr

residues. Some members of this sub-family are also lipid phosphatases such as PTEN which can dephosphorylate both lipids as well as proteins.

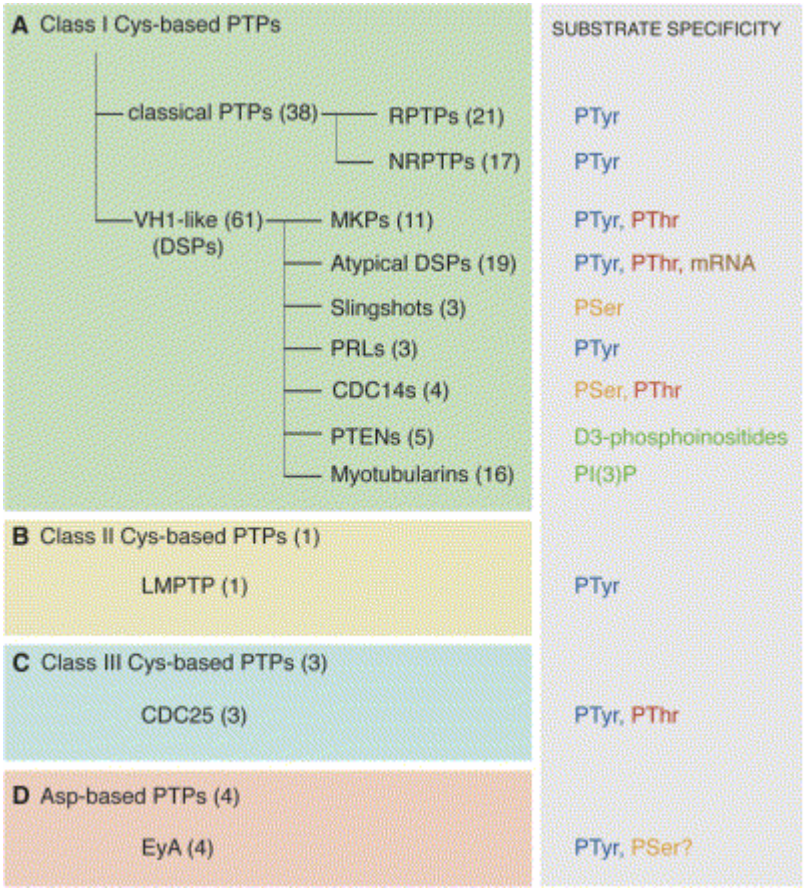


Fig. 1.2 Classification and substrate specificities of PTPs [taken from (Alonso, Sasin et al. 2004)].

The PTPs are characterized by the presence of conserved stretches of 240-250 amino acids (PTP domains) including the signature motif (VH**C**SXGXG**R**[T/S]G) which is essential for their action (Andersen, Mortensen et al. 2001). During PTP catalysis, the cysteinyl residue executes a nucleophilic attack (enhanced by the arginine residue in the signature motif) upon the oncoming phosphate moiety of the substrate, leading to formation of a thiophosphate intermediate. The depth of the cleft is set by an invariant tyrosyl residue thus allowing entry of only the longer phospho-tyrosine and not the shorter phospho-serine or phospho-threonine moieties. For efficient thiophosphate intermediate formation, the phenolic oxygen of the tyrosyl leaving group must be

protonated and a conserved aspartate residue (Asp 181 in PTP 1B) donates this proton thus serving as a general acid. Mutation of either the catalytic cysteine residue (into serine) or of the conserved aspartate residue (into alanine) completely abolishes the activity of the PTPs but still retains their ability to bind to substrates. Such PTP mutants are therefore helpful in identifying substrates by the so called "trapping" approach (Neel and Tonks 1997; Tonks and Neel 2001).

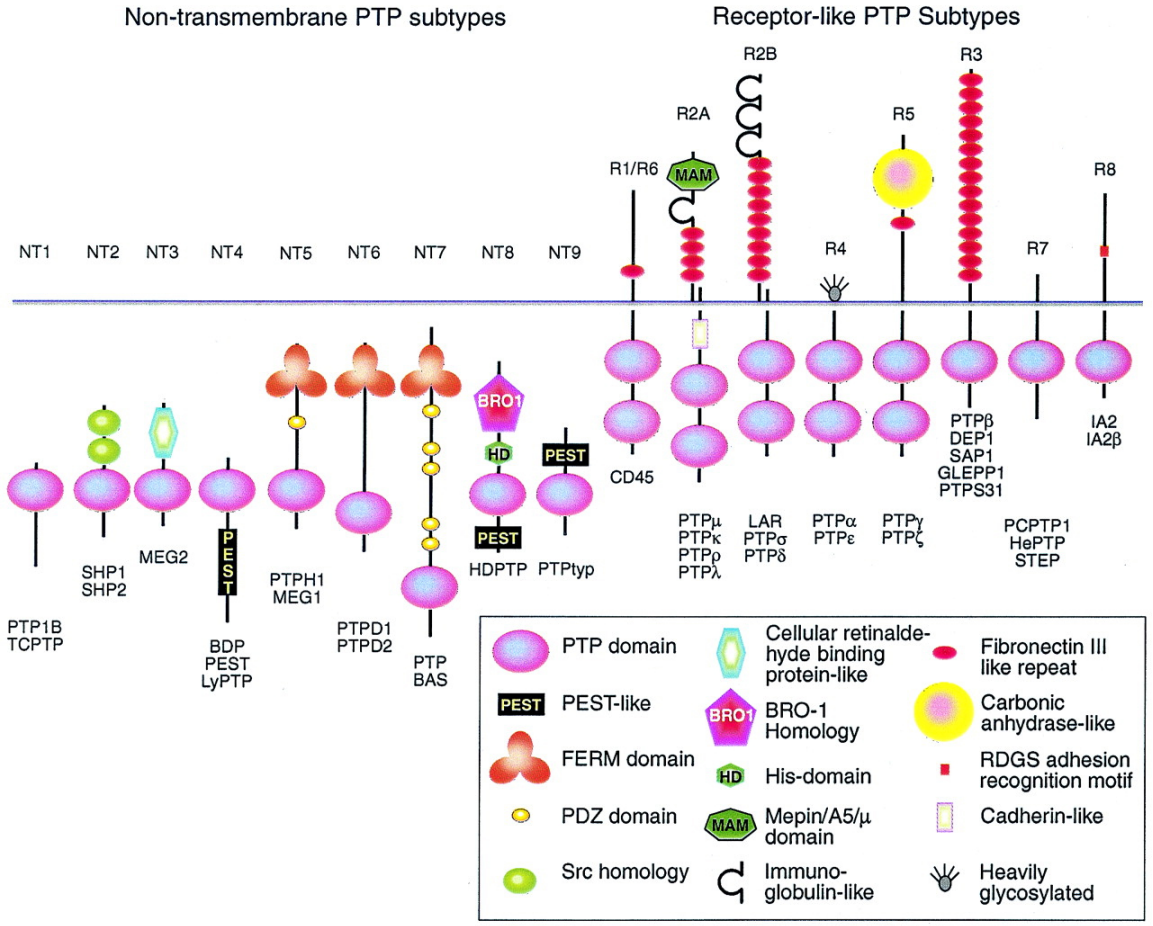


Fig. 1.3 Schematic representation of PTP family members [taken from (Andersen, Mortensen et al. 2001)].

These 38 "classical PTPs" are further divided into transmembrane, receptor like PTPs (RPTPs) and the intracellular, non-receptor PTPs (NRPTPs). RPTPs can be further subdivided into five types (Type I – V) based upon common features in their extracellular domains. Most RPTPs contain two PTP domains and usually the membrane proximal

(amino-terminal) of these two domains (D1) has significant enzymatic activity. The distal one (D2) has less if any activity and may aid in protein-protein interactions or in regulating the activity of D1. Intracellular non-transmembrane PTPs (NRPTPs) possess a single catalytic domain with flanking regions. They often contain protein-protein interaction domains that direct the enzyme towards specific intracellular locations or towards substrates (Li and Dixon 2000).

VH1-like DSPs are classified into MAP Kinase Phosphatases (MKPs) which are characterized by the presence of dual phospho-threonine and phospho-tyrosine specificity. The second group called “Atypical DSPs” are much smaller enzymes and have functions unrelated to MAP kinases. One outstanding example is PIR (DUSP11) which can dephosphorylate mRNA. The third group, “slingshots” is involved in the dephosphorylation and inactivation of Cyclin Dependent Kinases (Cdks). The last two subgroups, PTENs and myotubularins dephosphorylate phosphatidyl inositol phospholipids (Alonso, Sasin et al. 2004).

The class II PTP family is represented by a single gene in the human genome ACP1 which codes for 18 kDa Low Molecular phosphatase (LMPTP). However, related class II enzymes are widely present in prokaryotes. Class III PTPs comprise three cell cycle regulators, Cdc25A, Cdc25B, and Cdc25C which dephosphorylate Cdks at their inhibitory dually phosphorylated Thr-Tyr motifs, thereby activating them and driving the cells through cell cycle. Class IV Asp-based PTPs are represented by four “eyes absent” (Eya) genes. These PTPs are unique as they do not use catalytic cysteine as a nucleophile but an aspartic acid, in a metal dependent fashion. Eya PTPs are transcription factors and have phosphatase activity against both tyrosine and serine residues (Rayapureddi, Kattamuri et al. 2003). The members of the PTP family with their diverse structure are involved in the regulation of different processes ranging from development, axonal guidance, cell cycle, growth factor signaling, hematopoiesis, as well as liver and pituitary development (Van Vactor, O'Reilly et al. 1998). However due to constraint of space, emphasis will be laid here only on the functions of classical PTPs.

1.4.2. Regulation of PTP Activity

The activity of PTPs can be regulated by several mechanisms – autoinhibiton, ligand binding, phosphorylation, oxidation and dimerization.

1.4.2.1. Ligand binding

The extracellular domains of RPTPs suggest that they may participate in ligand mediated interactions. Homophilic interactions for some RPTPs (PTPμ, PTPκ) have also been reported (Brady-Kalnay, Flint et al. 1993; Sap, Jiang et al. 1994). The surface protein contactin has been shown to interact with PTPα and PTPβ/ζ (Peles, Nativ et al. 1995) and the extracellular matrix proteins tenascin and laminin-nidogen complex binds to PTPβ/ζ and LAR respectively (O'Grady, Thai et al. 1998). There are two reports showing modulation of PTP activity due to ligand binding. Inactivation of PTPβ/ζ by pleiotrophin and activation of DEP-1 by an unknown component of matrigel have been reported (Meng, Rodriguez-Pena et al. 2000; Sorby, Sandstrom et al. 2001).

1.4.2.2. Phosphorylation

Tyrosine phosphorylation of PTPs has been described for SHP1, SHP2, PTP1B, LM-PTP among others (Chiarugi, Taddei et al. 2005). SHP2 contains two C-terminal tyrosine residues (Tyr^{542}, Tyr^{580}). An *in vitro* study in which these phospho-tyrosines were replaced by non-hydrolysable analogs showed that phosphorylation at each of these position stimulates catalysis by three folds and even the double phosphorylation increased the activity to nine folds (Lu, Shen et al. 2003). SHP2 has also been shown to undergo tyrosyl phosphorylation following growth factor stimulation (Araki, Nawa et al. 2003). In the case of PTP 1B, the role of tyrosyl phosphorylation is controversial. Some studies showed an increase in the phosphatase activity following tyrosyl phosphorylation, (Dadke, Kusari et al. 2001) whereas a mouse model showed an opposing effect (Tao, Malbon et al. 2001). Phosphorylation of Tyr^{131} in LM-PTP by Src kinase has also been reported to increase its enzymatic activity (Chiarugi, Taddei et al. 2005).

1.4.2.3. Oxidation

Reactive Oxygen Species (ROS) are mainly produced by mitochondria and NADPH oxidases under different circumstances including as a consequence of various growth factor stimulation (Chiarugi and Cirri 2003). The cysteine residue in the signature motif

of the phosphatase displays an unusually low pKα which makes it susceptible to oxidation. Oxidation of this active site cysteine is coupled to its inactivation due to the formation of sulphenic acid (-SOH) derivative. This can be further oxidized to sulphinic acid ($-SO_2H$) and sulphonic acid ($-SO_3H$) forms which are usually irreversible (Tonks 2005). Structural analysis of PTP 1B revealed that the generation of sulphenic acid (-SOH) is followed by the formation of a cyclic sulphenylamide intermediate (a 5-atom ring structure in which a covalent bond is formed between the sulfur atom of the cysteine and the main chain nitrogen of the adjacent serine residue). This sulphenyl amide intermediate can be easily reduced by some cellular reducing agents such as GSH regenerating the active enzyme. Thus a transient inhibition of PTPs through the reversible oxidation of their catalytic cysteine can enhance the outcome of the signal (usually by hyper-phosphorylation of the RTK) (Salmeen, Andersen et al. 2003). Such a reversible oxidation mechanism has been demonstrated for PTP 1B during EGF and insulin signaling and for LM-PTP and SHP2 during PDGF signaling (Meng, Fukada et al. 2002; Meng, Buckley et al. 2004). Reversible oxidation has also been shown for DSPs such as Cdc25 and PTEN. In these cases, upon oxidation, the active site cysteine forms a disulfide bond with another vicinal cysteine leading to its inhibition (Lee, Yang et al. 2002).

1.4.2.4. Dimerization

Dimerization induced inactivation of PTPs has been shown for CD 45 and PTPα. The inhibitory dimerization involved D1-D1 interactions at least in the case of PTPα (Jiang, den Hertog et al. 1999). However crystal studies on CD 45 implicated that in this PTP D1-D1 interactions are not possible due to the spatial arrangement of D1 and D2 domains. Moreover structural analysis of transmembrane phosphatases of the LAR family has also emphasized that dimerizations induced inactivation is not an universal mechanism for the regulation of transmembrane phosphatases (Jiang, den Hertog et al. 1999; Nam, Poy et al. 1999).

1.4.3. Protein Tyrosine Phosphatases in Disease and Cancer

Studies using transgenic and knock out mice have revealed the involvement of PTPs in various kinds of physiological regulation and diseases. A few of them are discussed here.

1.4.3.1. PTPs in metabolism, development and immune system

PTP1B: $PTP1B^{-/-}$ mice showed reduced plasma glucose and insulin levels, obesity resistance and hyperphosphorylation of the insulin receptor. PTP 1B is therefore widely recognized as a candidate drug target. Metabolic regulation by PTP 1B does not only occur by its action on the insulin receptor (direct dephosphorylation). It also inhibits LeptinR signaling (Jak-2 dephosphorylation) thereby regulating lipid metabolism and energy expenditure (Elchebly, Payette et al. 1999; Cook and Unger 2002).

CD 45: Knock out mice show a variety of lymphocyte abnormalities including block in maturation of thymocytes and poor proliferative responses of peripheral T cells to TCR cross-linking. CD 45 is also necessary for normal antigen receptor signaling in both B and T cells and is potentially a good target for immunosuppressors (Byth, Conroy et al. 1996; Li and Dixon 2000; Hooft van Huijsduijnen, Walchli et al. 2002).

VE-PTP (Vascular-Endothelial PTP): VE-PTP is also referred to as PTPβ (see Fig. 1.3). It can dephosphorylate Tie-2 RTK, which responds to its ligand angiopoietin-1 (Fachinger, Deutsch et al. 1999). Signaling through this receptor antagonizes blood vessel leakage (anti-inflammatory). Hence VE-PTP could be exploited as a target in inflammation since its inhibition should block neutrophil and macrophage extravasations (van Huijsduijnen, Bombrun et al. 2002).

LAR: $LAR^{-/-}$ females are incapable of delivering milk and have significantly lower plasma levels of insulin and glucose, suggesting elevated insulin sensitivity (Schaapveld, Schepens et al. 1997; Ren, Li et al. 1998). Another LAR family member is PTPσ (see Fig. 1.3). Targeted deletion of PTPσ causes stunted growth, developmental delays and severe neurological disorders (Elchebly, Wagner et al. 1999). The third member of the LAR family is PTPδ (see Fig. 1.3). Deletion of PTPδ causes growth retardation, abnormal positioning of hind limbs and abnormal limb flexing (Uetani, Kato et al. 2000).

1.4.3.2. PTPs in cancer

Oncogenic signaling due to hyperactivation of tyrosine kinases is already well established. PTPs functions both as positive and negative mediators of signaling triggered by RTKs, integrins and cell-adhesion molecules indicating that they may either act as tumor suppressors or as oncoproteins. Some examples of tumor suppressive PTPs are DEP-1, SHP1 and GLEPP1 whereas oncogenic activity has been characterized for the mutational activation of SHP2.

PTPs as tumor suppressors

SHP1 (SH2 domain PTP): SHP1 is an antagonist of growth-factor signaling in epithelial and haemotopoietic cells. Motheaten (me/me) and motheaten viable (me^v/me^v) mice which either have no SHP1 protein or SHP1 protein with little catalytic activity exhibit hyperproliferation of hematopoietic cell lineages. Inactivation of SHP1 expression by promoter hypermethylation has been described in T-cell lymphoma, large cell lymphoma as well as in myeloma (Fig. 1.4(c)). SHP1 re-expression causes decreased phosphorylation of JAK3 and STAT3 (Chim, Fung et al. 2004; Ostman, Hellberg et al. 2006).

GLEPP1 (Glomerular Epithelial Protein-1): GLEPP1, encoded by the gene *PTPRO*, has a single intracellular catalytic domain and several extracellular fibronectin type III repeats (see Fig. 1.3). *PTPRO* promoter hypermethylation has been described in about 50% of human lung cancer and microsatellite-instability-associated colon cancers. Over-expression of GLEPP1 leads to reduced anchorage independent growth, proliferation and resistance to apoptosis (Motiwala, Kutay et al. 2004). Another important tyrosine phosphatase, DEP-1, which is structurally similar to GLEPP1 also plays an important role as a tumor suppressor and its role will be described in later sections.

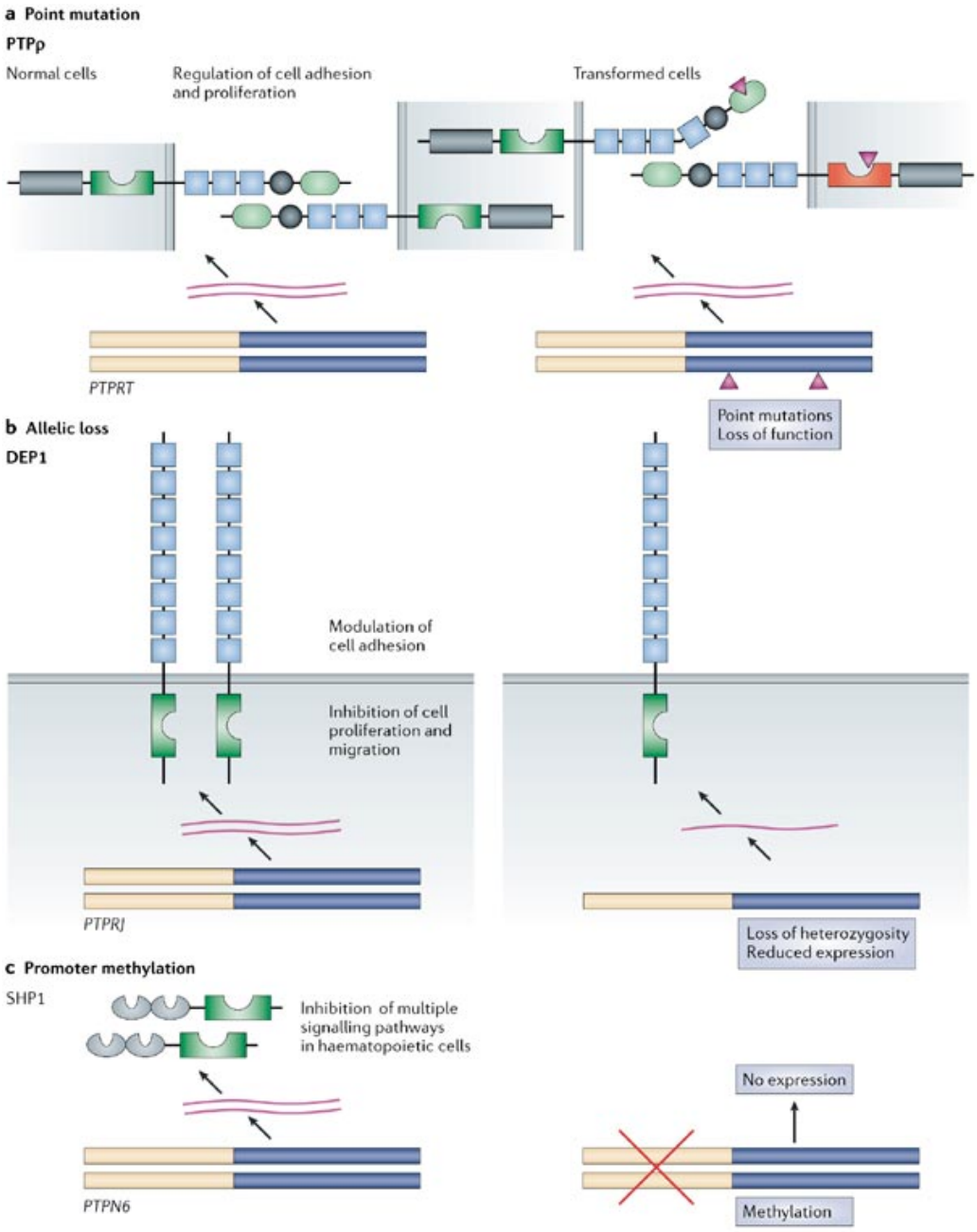


Fig. 1.4 Mechanisms of inactivation of PTPs with tumor suppressive activity. a) Point mutations in the coding region of PTPRT, which encodes the receptor-like PTP PTPρ. Several of these mutations have been predicted or shown to affect PTP-mediated cell–cell adhesion, or inhibition of cell proliferation. b) Allelic loss, but no point mutations, was found for PTPRJ, which encodes the receptor-like PTP DEP-1 in different types of carcinoma. c) Promoter silencing of the SHP1-encoding gene, PTPN6, by methylation occurs frequently in lymphoma and leukemia. In the figure, the promoter region and coding region of genes are indicated in yellow and blue, respectively. mRNA is symbolized in pink. At the PTP protein level, active states are in dark green and inactive states are in red [taken from (Ostman, Hellberg et al. 2006)].

PTEN (phosphatase and tensin homology deleted on chromosome ten): PTEN, has dual specificities as it can dephosphorylate both lipids (3-phosphoinositides) as well as proteins. Initially, based on the sequence homology it has been assigned as a protein tyrosine phosphatase, but later on it has been found that its primary substrate is a phosphorylated lipid – it catalyses the dephosphorylation of phosphatidylinositol-(3,4,5)-triphosphate (PIP_3) to phosphatidylinositol-(4,5)-diphosphate (PIP_2). As a PIP_3-phosphatase, PTEN has emerged as a principal antagonist of PI3K signaling. Germline mutations of PTEN lead to Cowden Syndrome which is associated with the development of malignant tumors. Somatic mutations of PTEN were also documented in a variety of malignant tumors at both advanced and early stages (Sulis and Parsons 2003). Moreover *Pten*$^{+/-}$ mice have also been shown to develop colonic adenomas and lymph node hyperplasia at a higher frequency because of haploinsufficiency (Podsypanina, Ellenson et al. 1999). Even though its function as a lipid phosphatase is well established, there are also reports showing that PTEN can dephosphorylate Focal Adhesion Kinase (FAK) and Shc, thereby regulating cellular motility and directionality (Wishart and Dixon 2002).

PTPs as oncoproteins

SHP2: SHP2 transduces mitogenic and promigratory signals from various types of receptors. In the absence of upstream stimulation, SHP2 is in an inactive state by interaction of its N-terminal SH2 domain with the catalytic PTP domain. Activation of surface receptors and subsequent phosphorylation, creates SHP2 binding sites in RTKs or in scaffolding adaptor proteins such as GAB2 (Grb2 associated binder 2) leading to SHP2 recruitment. Activated SHP2 enhances signaling pathways leading to enhanced Erk1/2, Akt or STAT5 activation. It has been shown that SHP2 dephosphorylates a GAB1 YXXP motif which is a binding site for Ras-GTPase activating protein (RASGAP) thereby preventing its action (Montagner, Yart et al. 2005). It has also been shown to dephosphorylate the docking site of Csk in a transmembrane glycoprotein; PAG/Cbp. Csk is a negative regulator of Src Family Kinases (SFKs) by phosphorylating them at their terminal inhibitory domain. Thus by dephosphorylating PAG, SHP2 displaces Csk, allowing an enhanced SFK activation leading to sustained Erk activation (Zhang, Yang et al. 2004).

SHP2 mutations are found in 50% of Noonan syndromes (dominant, hereditary) and 30% of juvenile myelomonocytic leukemia (JMML) as well as in some somatic cases of Acute Myeloid Leukemia (AML) and Acute Lymphoblastic Leukemia (ALL) (Tartaglia, Mehler et al. 2001). It has also been detected at low frequency in solid tumors such as lung, colon carcinoma and melanoma (Bentires-Alj, Paez et al. 2004). These mutants possess increased sensitivity for the corresponding ligands of upstream receptors. Activation of downstream signaling events occurs by similar mechanisms as that of wild type SHP2 but to supraphysiological levels and in a more sustained manner. Altered signaling may also be a result of the modified substrate selectivity of SHP2 mutants (Keilhack, David et al. 2005).

Cdc25: Cdc25s comprise three PTPs (see Fig. 1.2) that activate cyclin dependent kinases. Among these, Cdc25B is over-expressed in approximately 30% of breast cancers and is being explored as a drug target (Hooft van Huijsduijnen, Walchli et al. 2002).

Prl-3: Prl-3 is a dual specificity protein tyrosine phosphatase and specifically amplified and over expressed only in metastases but not in their originating tumors. Its level is significantly lower in normal, and in adenomas or primary cancerous tissue suggesting that it is necessary only for the metastasis and could be exploited as a drug target (Saha, Bardelli et al. 2001).

Some other PTPs such as PTPα, PTPε can activate Src family kinases (SFKs). Based on the data from some mouse models and some over expression studies in certain human tumors, they have also been proposed to promote cancers.

1.5. Cancer

Cancer arises owing to the accumulation of mutations in critical genes. It is characterized by the uncontrolled division of cells that leads to the formation of tumorous tissue. Genomic instability is one of the hallmarks of cancer. The mutations that are involved can be broadly classified into two major groups - gain of function mutations in proto-oncogenes and loss of function mutations in tumor suppressor genes. Progressive

mutations lead to the acquisition of several features - uncontrolled proliferation (requires exit from senescence or cell cycle arrest), resistance to apoptosis (programmed cell death) and also invasion of other tissues. This latter phenomenon is referred to as metastasis and involves the migration of tumor cells from their initial site to a distant site where they adhere and initiate a new tumor. Hence, several properties of the cell determine the cancer phenotype - altered proliferation, apoptosis, migration and adhesion (Hanahan and Weinberg 2000).

1.5.1. Proliferation

Various mitogenic factors including growth factors and cytokines can stimulate the proliferation of cells. Usually proliferation is initiated by the binding of the growth factor to its receptor, leading to receptor dimerization, activation and phosphorylation of downstream targets. One of the essential signaling pathways is the MAP kinase pathway. It comprises RTK signaling to the adaptor protein Growth Factor Receptor Bound 2 (Grb2), which then signals to Son of Sevenless (SOS). SOS, being a Ras Guanine nucleotide Exchange Factor (GEF), activates Ras, which leads to the activation of Raf, MEK (MAP/Erk Kinase) and Erk1/2. The latter enters the nucleus activating a set of transcription factors triggering proliferation (Hunter 2000). Activating mutations in any of the genes in this pathway leading to hyperactivation can cause cancer (Blume-Jensen and Hunter 2001). Therefore these genes are referred to as proto-oncogenes. To date, several genes in this pathway have been reported to play a role in cancer.

They can be broadly divided into

(a) Genes encoding secreted growth factors which act as ligands for RTKs. Examples of this class are Platelet Derived Growth Factor-beta (PDGF-B), Fibroblast Growth Factor (FGF) and Wingless (wnt-1).

(b) Mutation or hyperactivation of transmembrane growth factor receptors (RTKs). Few examples include Epidermal Growth Factor Receptor (EGFR), Fms like tyrosine kinase-3 (Flt-3) and c-Kit.

(c) Mutation or hyperactivation of cytoplasmic signaling molecules. Typical examples are members of the Ras GTPase family, c-Src and B-Raf.

(d) Hyperactivation by over expression of nuclear proteins such as c-myc and members of Signal Transducer and Activators of Transcription (STAT) family.

1.5.2. Apoptosis

It is also referred to as "programmed cell death" and helps to eliminate the unwanted or damaged cells in the organism. When a cell is under stress conditions such as starvation, UV-irradiation, DNA damaging events, apoptosis can be induced. There are several mechanisms of triggering apoptosis. It can be initiated by either ligand mediated (Fas L) or ligand independent pathways. In the latter case, mitochondria play a crucial role by releasing cytochrome C into cytoplasm. All pathways activate a group of proteases called caspases (Reed 1999). Apoptosis also affects the nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP-1). Upon induction of apoptosis, the 116 kDa PARP-1 is cleaved into an 89 kDa and a 24 kDa fragment and acts as a marker for the detection of apoptosis. (Soldani and Scovassi 2002). Many tumor suppressor genes promote apoptosis. Mutation or inactivation of these genes makes the cells resistant to apoptosis, thus favoring tumorigenesis.

However there are other sets of genes promoting cell survival, important of them is Protein Kinase B (PKB) also referred to as Akt. Activation of RTKs by ligands creates docking sites for a set of proteins leading to their recruitment. Phosphoinositide-3-kinase (PI3K) is one among them and recruited via binding of the SH2 domains of its regulatory subunit (p85) resulting in its activation. 3′-phosphoinositides generated by activated PI3K mediate the membrane recruitment of PKB/Akt from the cytosol via its PH domains changing the kinase from an inactive to an active state. PKB/Akt is then dually phosphorylated on Thr^{308} (by PDK1)and Ser^{473} (by an unidentified Ser^{473} kinase) (Brazil and Hemmings 2001). Activated PKB/Akt then phosphorylates a set of proteins promoting cell survival. PKB/Akt phosphorylates the pro-apoptotic protein BAD as well as caspase-9 thereby directly blocking apoptosis. It was also shown to phosphorylate I Kappa Kinase β (IKK β - negative regulator of NFκB), Mdm2 (ubiquitin ligase for the tumor suppressor p53), Forkhead family of transcription factors, CREB (cyclic AMP response element binding proteins), YAP (Yes associated protein) bringing a change in the cell survival (Song, Ouyang et al. 2005). Phosphatases such as PP2A can dephosphorylate PKB/Akt and can antagonize its functions.

1.5.3. Migration and Adhesion

Cell migration and adhesion are two tightly coupled processes which are critical for normal development, physiology and also cancer progression (particulary metastasis). They are in turn regulated by phosphorylation. Two types of adhesion can be distinguished: adhesion of cells to the underlying extracellular matrix, and adhesion between adjacent cells (Sallee, Wittchen et al. 2006). Adhesion of the cells to the underlying matrix is mediated by integrins and is usually initiated by the formation of focal complex at the cell periphery. Focal complexes are short lived and turn into focal adhesions which can be classified either into "classical" focal adhesions (located at cell periphery with high level of phospho-tyrosine proteins) or "fibrillar" focal adhesions (located at central positions with little or no phospho-tyrosine) (Zaidel-Bar, Ballestrem et al. 2003). Cell-cell junctions are usually classified into tight junctions (zonula occludens), adherens junctions (zonula adherens - formed by cadherins, catenins and linked to actin cytoskeleton) and desmosomes (macula adherens) (Cavallaro and Christofori 2004).

Cell migration requires a series of repetitive, integrated processes to produce coordinated cellular movements. It can be briefed in five steps. The first step is the protrusion of the cell. The cell extension can either be a lamellipodium (broad and thin mediated by Rac GTPase (Guanosine triphosphatase)) or a filopodium (smaller and finger-like sharp protrusions mediated by Cdc 42). The second step is the attachment of these protrusions to the extracellular matrix (ECM) by the formation of focal complexes, which turn into focal adhesions thus enabling integrins to connect the actin cytoskeleton to the ECM. The next event in the migratory process is the cell-body translocation which is brought about by actomyosin contraction. The last two phases in cell migration are the release of cell contacts at the rear of the cell and recycling of membrane receptors to the front (Larsen, Tremblay et al. 2003). Thus migration of cells requires a constant turnover of focal adhesions. The molecular events in this process can be briefed as follows (see Fig. 1.5): following integrin engagement to the ECM, Focal Adhesion Kinase (FAK) is recruited and is activated through autophosphorylation at Tyr^{397}. FAK then phosphorylates the adaptor protein paxillin at Tyr^{118}. The PTK Src is also recruited and activated by dephosphorylation at Tyr^{529} (initiated by a PTP such as PTPα or PTP1B) (Freiss and

Vignon 2004). Autophosphorylation of Src at Tyr^{418} activates it and leads to phosphorylation of other tyrosine residues in FAK. This results in docking sites for proteins like p130cas and Grb2. p130cas activates Rac through the adaptor protein Crk and the Rac GEF Dock 180 leading to lamellipodia formation. Grb2 leads to activation of Erk and Myosin Light Chain Kinase (MLCK) which is necessary for cell body translocation (Webb, Donais et al. 2004; Nishiya, Kiosses et al. 2005). Some PTPs can oppose Rac activation (PTP-PEST, PTEN). The formation/turnover of focal adhesions is regulated by PP2A, SAP1, PTP-PEST and MLCP which generally act to inhibit migration, whereas phosphatases such as PTP1B, SHP2 and PTPφ enhance migration (Larsen, Tremblay et al. 2003).

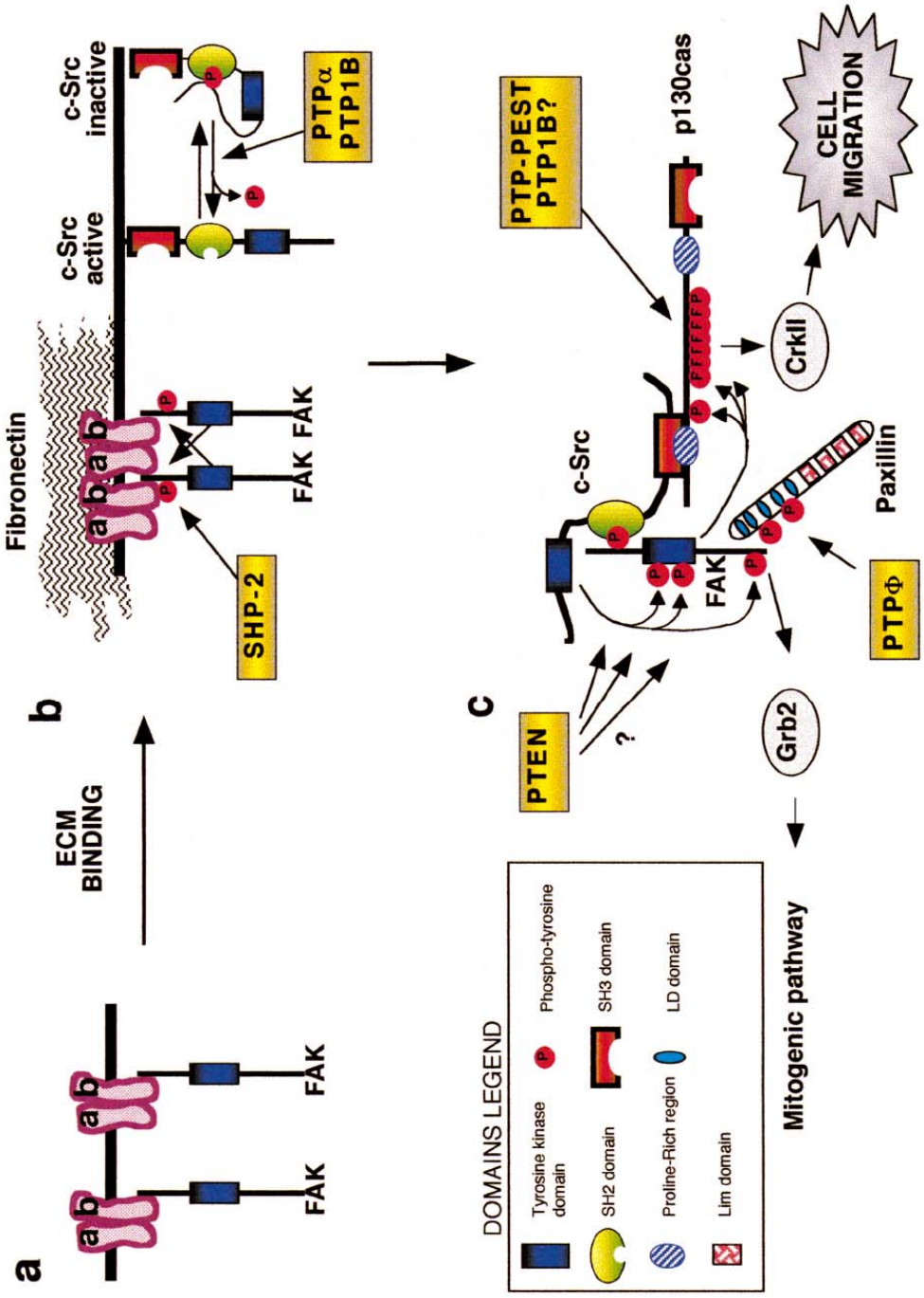


Fig. 1.5 FAK-dependent signaling pathways. a) Status of FAK in cells kept in suspension. b) Binding of the cell to extracellular matrix proteins (ECM) induces phosphorylation and activation of FAK. c-Src is then activated and can bind the phosphorylated Y^{397} on FAK. c) Activated c-Src phosphorylates FAK and increases its catalytic activity. It also bridges p130cas and FAK, allowing signaling downstream of the adaptor protein. Activated FAK also associates with the paxillin through a LD domain [taken from (Angers-Loustau, Cote et al. 1999)].

1.5.4. Angiogenesis

Angiogenesis is defined as the process of the growth of new blood vessels. It is very essential during organogenesis as the oxygen and nutrients supplied by the vasculature are crucial for cellular function and survival. It has also been implicated in tumorigenesis since vascularization of tumors is required to nourish the hyperproliferating cells. In general, there are both positive and negative mediators of angiogenesis. Important are Vascular Endothelial Growth Factor (VEGF) and basic fibroblast growth factor (bFGF), which act positively whereas thrombospondin-1 or β-interferon act in a negative manner. Also, the transition from quiescent to sprouting capillaries is often accompanied by a change in the class of integrin expressed (Hanahan and Weinberg 2000). Generally the hyperproliferating cells in a tumor have a hypoxic environment. Such cells usually induce hypoxia-inducible factor-1α (HIF-1α), a key transcription factor that promotes angiogenesis (Harris 2002). Attempts are already underway in curtailing cancerous growth by targeting different angiogenic factors such as HIF-1α or VEGF (Semenza 2003). Avastin, a monoclonal antibody targeting VEGF is already on the market for the treatment of colon cancers.

1.6. Colon Cancer

Colorectal cancer is one of the common cancers and stands fourth in occurrence including both men and women accounting for 655,000 deaths annually worldwide (www.who.int). The incidence is generally higher in the developed countries than in the developing countries. Both environmental and hereditary factors contribute to the development of colorectal tumors (Rupnarain, Dlamini et al. 2004). The different stages of cancer progression were best studied with colon cancer (Fig. 1.6). Initially the hyperplastic epithelium appears normal, advancing to adenomatous polyps at different stages of dysplasia (by acquiring mutations in critical genes), later to a non-invasive, and finally to an invasive carcinomatous phenotype (Weinberg 1991).

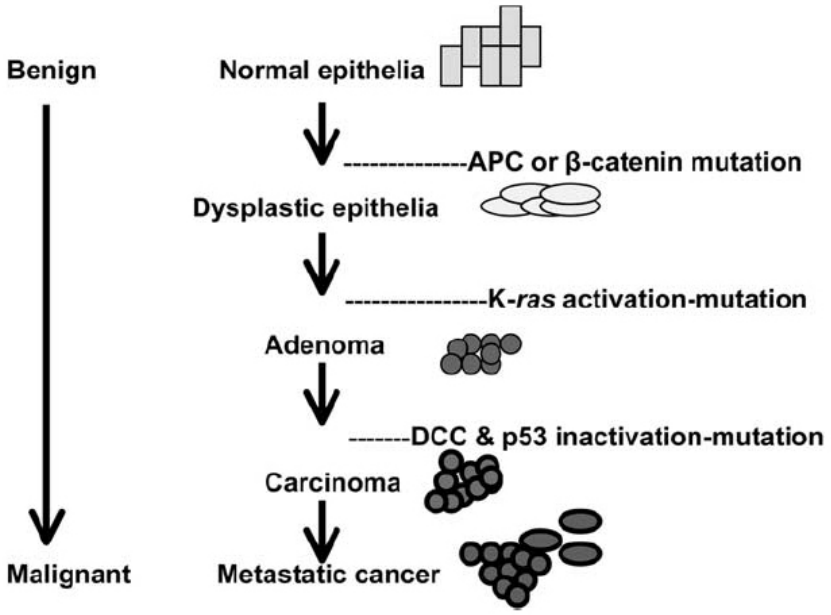


Fig. 1.6 Schematic representation of the genetic changes involved in colon cancer development. DCC: deleted in colorectal cancer, K-ras: Kirsten-ras [from (Rupnarain, Dlamini et al. 2004)].

1.6.1. Critical Genes and Pathways in Colon Cancer

Genes that are critical and frequently found to be mutated in colon cancer are the K-ras, Adenomatous Polyposis Coli (APC), β-catenin, p53 and MMR (responsible for DNA mismatch repair). B-Raf point mutations have also been recently reported in a subset of colon cancers (Davies, Bignell et al. 2002). The major pathways that are altered in relation to colon cancer and drive its progression are: Erk pathway (enhances proliferation), Wnt signaling pathway (stabilization of β-catenin and activation of TCF-LEF driven genes), increased survival signal/resistance to apoptosis (due to mutations in p53, Rb, PTEN), JAK-STAT pathway (generally activated by cytokine receptors and hyperactivation of STAT 3), TGF β (Transforming Growth Factor beta)-SMAD pathway. However, other genes including that of Cyclo-Oxygenase 2 (COX-2), Matrix Metalloproteinases (MMPs), Hypoxia Inducible Factor 1 alpha (HIF 1α), Deleted in Colorectal Cancer (DCC) and Mutated in Colorectal Cancer (MCC) are also relevant in the progression of colorectal cancer.

1.6.2. Therapy and Treatments

Current treatments of colon cancer are the surgical removal in early cases, chemotherapy and radiation therapy. Chemotherapy is based on the action of chemicals particularly affecting DNA replication. Some of them are 5-fluorouracil, raltitrexed (inhibits thymidylate synthase), irinotecan (topoisomerase I poison) and oxaliplatin (bifunctional platinum alkylator of DNA) (Tebbutt, Cattell et al. 2002). Recently, targeted therapies using monoclonal antibodies have also been developed. Two of them were already approved for the treatment of colorectal cancer in 2004 by the Food and Drug Administration (FDA). Bevacizumab (Avastin) targets the Vascular Endothelial Growth Factor (VEGF) blocking angiogenesis thereby starving the tumor. Cetuximab (Erbitux), a monoclonal antibody against the EGF receptor inhibits signals emanating from EGFR (Adams and Weiner 2005).

1.6.3. The Role of Diet in Colon Cancer

The high incidence of colon cancer in the developed and western countries is sometimes correlated with the food habits. Studies have shown that diet rich in refined grain and red meat significantly increased the risk of colorectal cancer (Levi, Pasche et al. 1999) whereas, a diet rich in vegetables, fruits and whole grain consumption decreased the risk of polyp formation. These potential protective effects may result from the high levels of dietary fibre, antioxidants (beta-carotene, vitamin C and polyphenols) or other anti-carcinogenic constituents (protease inhibitors and phytoestrogens) (Witte, Longnecker et al. 1996).

1.6.3.1. Polyphenols

Polyphenols are the most abundant antioxidants in our diets. The main classes of polyphenols are phenolic acids (mainly caffeic acid which occurs in foods mainly as an ester with quinic acid called chlorogenic acid) and flavonoids. Flavonoids are sub-divided into several classes: flavonoles (myricetin, fisetin, quercetin, kaempferol – abundant in normal diet and present in onions), isoflavones (daidzein, genistein – soy and dry beans), flavonols (catechins – present in tea also as their galloylated derivatives), flavanones (taxifolin, naringenin and hesperitin – citrus fruits) and flavones (luteolin, wogonin and apigenin - present in sweet red pepper and celery) (Fig. 1.7). In addition, other classes

such as anthocyanins (pigments of red fruits and present in red wines), proanthocyanidins and stilbenes (reservatrol – present in wine and has anticarcinogenic properties) also belong to the group of flavonoids (Tapiero, Tew et al. 2002).

Several studies have emphasized the protective effects of various flavonoids in relation to colon cancer. The core structure of flavone (2-phenyl-4H-1-benzopyran-4-one) alone has been shown to affect proliferation, differentiation and apoptosis differently between transformed colon cancer cell line (HT 29) and non-transformed colon cells. These effects were also correlated to a change in the mRNA levels of cell cycle and apoptosis related genes including cyclo-oxygenase 2 (COX-2), NF κB and bcl-X_L (Wenzel, Kuntz et al. 2000). Different flavonoids can also inhibit the expression of COX-2 (usually upregulated in colon cancer) to different extents, depending on their chemical structure (Mutoh, Takahashi et al. 2000). Flavonoids such as epigallocatechin gallate (EGCG), piceatannol (natural analog of reservatrol) have also been shown to inhibit cell cycle progression in CaCo-2 cells, a colon cancer cell line (Salucci, Stivala et al. 2002; Wolter, Clausnitzer et al. 2002).

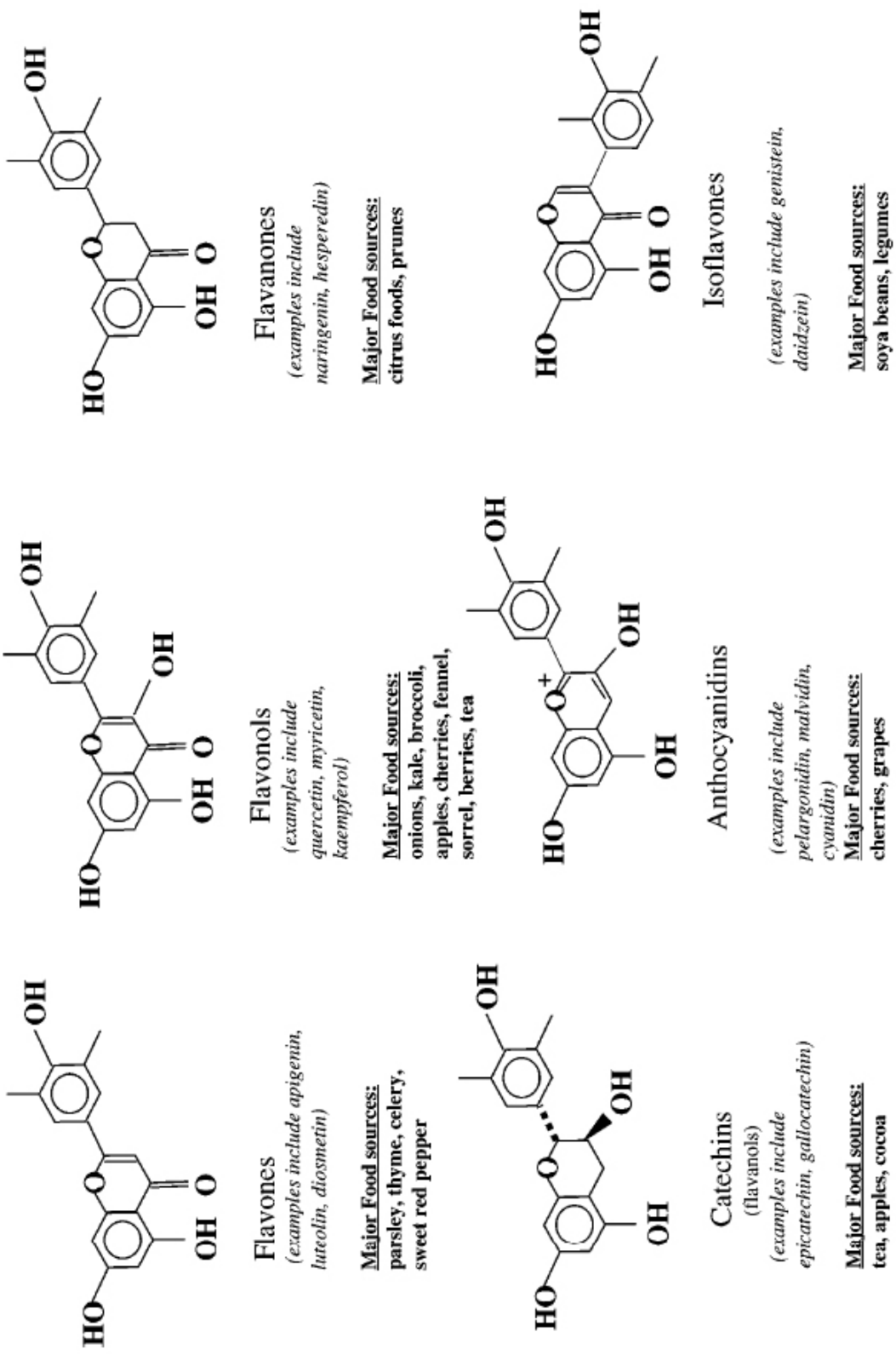


Fig. 1.7 Major subclasses of flavonoids, their structure and food sources [taken from (Ross and Kasum 2002)].

1.6.3.2. Apples

Apple is one of the common fruits. It is rich in polyphenols including epicatechin, caffeic acid, phloretin-2'-glucoside (phloridzin), phloretin-2'-xyloglucoside, 3-hydroxy-phloridzin, quercetin-3-arabinoside, quercetin-3-xyloside, quercetin-3-galactoside, quercetin-3-glucoside (isoquercetin) and quercetin-3-rhamnoside (Lu and Foo 1997). Apple contains significant amounts of anti-oxidants which have the capacity to scavenge radicals (Lu and Foo 2000). Recently it has been shown that an apple polyphenol extract (AE) contains inhibitors of EGFR. Cell treatments with AE reduced EGF induced Erk activity and inhibited proliferation (Kern, Tjaden et al. 2005). AE has also been shown to reduce oxidative damage in human colon cells (Schaefer, Baum et al. 2006; Schaefer, Baum et al. 2006). Another study has shown that AE inhibits the growth of HT 29 colon cancer cells. Furthermore, using a cDNA microarray based mRNA expression analysis, it has also been shown that AE can upregulate several genes (GSTP1, GSSTT2, MGST2, CYCP4F3, CHST5, CHST6 and CHST7) involved in detoxification (Veeriah, Kautenburger et al. 2006). Interestingly, the beneficial effects of AE with respect to colon cancer development were shown by three independent groups *in vivo* in rats. AE prevented both oxidative-induced damage in gastric epithelial cells, MKN 28 (*ex vivo*) as well as indomethacin induced injury in rats (*in vivo*) (Graziani, D'Argenio et al. 2005). In another study, the chemopreventive effects of AE were assessed. Rats were intraperitoneally injected with the carcinogen 1,2-dimethylhydrazine (DMH), and were given either water or apple juices for 7 weeks starting one week before DMH treatment. Apple juice drinking rats showed a reduction of genotoxic damage in mucosa cells, crypt cell proliferation as well as reduction in the number of aberrant crypt foci (ACF) (Barth, Fahndrich et al. 2005).

1.6.3.3. Butyrate and Green tea

Another interesting compound of dietary origin is butyrate. Butyrate is produced in the colon as a fermentation product from various precursors (Beyer-Sehlmeyer, Glei et al. 2003). It is known for its antiproliferative activity on colon cancer cells in which it also induces apoptosis and promotes differentiation. It also acts as a survival factor (Hague, Singh et al. 1997) and a nutrient for non-transformed cells (Hague, Butt et al. 1996).

Differential patterns of gene expression were also reported for human colon cells treated with butyrate. Some of them are the enhanced expression of different isoenzymes of Glutathione-S-Transferases (GSTs), which may enhance cellular chemoprotection (Pool-Zobel, Selvaraju et al. 2005). Also epigallocatechine-3-gallate (EGCG), a major component of green tea and related constituents has various anticancer effects, including inhibition of proliferation of human colon cancer cells (Shimizu, Deguchi et al. 2005; Shimizu, Deguchi et al. 2005).

1.7. Colon Cancer and Tyrosine Phosphatases

Since mutations of genes encoding proteins that regulate tyrosine phosphorylation can contribute to cancer, the tyrosine phosphatase gene superfamily was analysed for mutations in human cancers. This study revealed 83 somatic mutations in six different PTPs (*PTPRF, PTPRG, PTPRT, PTPN3, PTPN13 and PTPN14*) affecting 26% of colorectal cancers. A number of these mutations were also found in a smaller fraction of lung, breast and gastric cancers. Some of the mutations resulted in truncated proteins lacking phosphatase activity. One of these PTPs, *PTPRT* was studied in detail. Biochemical studies revealed that all the five identified missense mutations of *PTPRT* resulted in reduced phosphatase activity. The authors of this study further showed that over expression of this PTPRT inhibited cell growth in colony assays. Based on these findings, it was proposed that the mutated PTPs may aid in tumor suppression (Wang, Shen et al. 2004).

1.7.1. Protein Tyrosine Phosphatase DEP-1 and Cancer

Density Enhanced Phosphatase-1 (DEP-1) was initially cloned independently by two different groups and also referred to as PTPRJ, PTP-η, CD 148 (Honda, Inazawa et al. 1994; Ostman, Yang et al. 1994). As the name indicates, its expression is increased upon high cell density in a number of cell lines. DEP-1 is a glycosylated, transmembrane protein with a molecular weight of 180-220 KDa (1337 aminoacids). It has eight fibronectin repeats on the extracellular side (940 aa), a transmembrane region and a single catalytical domain (341 aa) on the intracellular side which harbors the phosphatase activity. Sequence analysis of the catalytic domains revealed that the closest relationships

are to the other members of class III RPTPs particularly PTPβ and SAP-1 (see Fig. 1.3). The chromosomal location is at 11p11.2. DEP-1 is a ubiquitously expressed protein ranging from lymphoid organs (B cells, T cells, granulocytes, macrophages, dendritic cells and thymocytes) to non-lymphoid organs (epithelial cells, fibrocytes, melanocytes and schwann cells). Its expression is absent in muscular tissue, adipose tissue, axons and ganglions of peripheral nervous system, neurons and microglia of the central nervous system (Autschbach, Palou et al. 1999). Several lines of evidences suggest DEP-1 as a tumor suppressor protein.

Crossing of the mouse strains STS/A (colon cancer susceptible) with BALB/cHeA (colon cancer resistant) revealed the association of two loci namely *Scc1* and *Scc2* with colon cancer susceptibility (Moen, Groot et al. 1996). Further mapping and cloning of this quantitative trait locus *Scc1* identified the phosphatase *Ptprj* as the underlying gene. *PTPRJ* is also frequently found to be lost in human cancers of lung, colon and breast. (Ruivenkamp, van Wezel et al. 2002). Another recent study about loss of heterozygosity (LOH) in human aberrant crypt foci (ACF) has revealed that a LOH is an early event (even before APC or β-catenin mutations). In a majority of cases a LOH was found at the chromosomal location 11p11, the chromosomal region containing *PTPRJ* (Luo, Shen et al. 2006).

1.7.1.1. DEP-1 in other cellular models

Breast

Constitutive over-expression of DEP-1 in breast cancer cell lines reduced the colony formation by 3-5 folds; whereas upon induction with an inducible metallothionin promoter, cell growth was greatly inhibited (5-10 fold). DEP-1 was also found to be induced in breast carcinoma cells (SKBr-3) undergoing differentiation upon treatment with sodium butyrate (Keane, Lowrey et al. 1996).

Thyroid

In the case of thyroid carcinomas, the DEP-1 protein levels are shown to be drastically reduced in tumor samples when compared to that of normal thyroid tissues. Also the expression of the rat variant DEP-1 (rPTPη) in transformed cell lines induced

morphological reversion, upregulation of differentiation markers and a significant reduction in proliferation. These effects were attributed to increased $p27^{kip1}$ protein levels. (Trapasso, Iuliano et al. 2000). Furthermore, the growth of xenograft tumors in athymic mice from anaplastic thyroid carcinoma cells transduced with the Ad-r-PTPη virus were drastically reduced (Iuliano, Trapasso et al. 2003). Recently it has also been shown that DEP-1 co-immunoprecipitates with the kinae Rearranged during Transfection (RET, an RTK implicated in thyroid tumors), dephosphorylates its autophosphorylation sites and thereby antagonizes RET signaling (Iervolino, Iuliano et al. 2006).

Endothelium

In the case of endothelial cells, DEP-1 is found in the complex of VE-Cadherin-β-catenin where it can dephosphorylate VEGFR-2, thus reducing proliferation and potentially mediating contact inhibited cell growth (Lampugnani, Zanetti et al. 2003). Recently it has been shown that a bivalent form of a DEP-1 activating monoclonal antibody against the extracellular domain blocks angiogenesis in mouse cornea by mediating G_0/G_1 mediated cell cycle arrest. An activation mechanism involving ectodomain induced oligomerisation of DEP-1 has been proposed (Takahashi, Takahashi et al. 2006). Thus DEP-1 is shown to play a pivotal role in endothelial cells related to angiogenesis and vasculogenesis (Argraves and Drake 2005).

Glioma cells

In glioma cells, the anti-proliferative effect of somatostatin appears to be mediated through the expression as well as the activity of DEP-1 by antagonizing Erk½ activation (Massa, Barbieri et al. 2004).

Pancreas

In pancreatic cancer cells, restoration of DEP-1 expression inhibits their proliferation. Cell cycle analysis revealed a substantial population of DEP-1 expressing cells in sub-G1 phase indicating the apoptotic potential of DEP-1 (Trapasso, Yendamuri et al. 2004).

Fibroblasts

Controversial results have been obtained in the case of fibroblasts. Previous data from our group showed that inducible over expression of DEP-1 inhibits migration, enhances cell-matrix adhesion and plays no role in proliferation. These effects were mediated by

antagonizing Ras-MAPK pathway as well as PLCγ- and Src-activation (Jandt, Denner et al. 2003). However, another group has shown that inducible over expression of DEP-1 inhibits proliferation and migration. Moreover, in these experiments cell-matrix adhesion was reduced upon DEP-1 expression due to defective turnover of focal contacts; whereas cell-cell adhesion was enhanced and associated with re-distribution of cadherin and catenin junctions (Kellie, Craggs et al. 2004).

T cells

In the case of T cells, DEP-1 over-expression inhibited activation of the "Nuclear Factor of Activated T cells" (NFAT) following T cell receptor stimulation. A mechanism involving the dephosphorylation of phospholipase Cγ1 (PLCγ1) and Linker for Activation of T cells (LAT) has been proposed (Baker, Majeti et al. 2001). Another study has proposed a mechanism in which CD 148 (i.e., DEP-1), due to its large extracellular domain is excluded from the immunologic synapse [T cell receptor (TCR) of T cells with Antigen Presenting Cells] thereby blocking access to the substrate TCR. Upon T cell-antigen presenting cell disengagement, CD 148 can then access and dephosphorylate TCR leading to down-regulation of signaling (Lin and Weiss 2003).

1.7.1.2. Substrates

Several studies involving over-expression have indicated some potential substrates of DEP-1. Site selective dephosphorylation of PDGFR-β has been shown in Porcine Aortic Endothelial (PAE) cells (Kovalenko, Denner et al. 2000). $pTyr^{1021}$, a PLCγ binding site is preferred rather than $pTyr^{857}$ (kinase domain) or $pTyr^{562}$ (Persson, Engstrom et al. 2002). Studies with DEP-1 "trapping" (DA) mutants have revealed HGFR (Met) as another possible substrate. Again site selective dephosphorylation is emphasized. DEP-1 preferentially dephosphorylated the Gab1 binding site ($pTyr^{1349}$) and a C-terminal site ($pTyr^{1365}$) rather than the activation loop / kinase domain ($pTyr^{1230/1234/1235}$) hence presumably serving as a specific modulator rather than a simple on-off switch for the RTK. A genetic study in *C.elegans* has shown that the *C.elegans* homolog of DEP-1 can negatively regulate LET-23 EGFR signaling in the developing vulva (Berset, Hoier et al. 2005). In the case of endothelial cells, DEP-1 has been shown to be present in a complex of VE-cadherin and β-catenin where DEP-1 dephosphorylates VEGFR-2. In glioma cells,

DEP-1 has been shown to antagonize activation of Erk1/2. It has also been proposed that DEP-1 can directly interact and dephoshorylate Erk1/2 *in vitro*. Another study has suggested that DEP-1 can interact with c-Src and can dephosphorylate its inhibitory C-terminal ($pTyr^{529}$) site. This leads to the activation of c-Src which enhances adhesion and causes hyperphosphorylation of focal adhesion proteins such as FAK and paxillin (Pera, Iuliano et al. 2005). DEP-1 has also been shown to interact with p120catenin, a component of adherens junctions (in A431 cells) thereby presumably playing a role in the regulation of cell-cell contact (Holsinger, Ward et al. 2002). A validated ligand for DEP-1 is yet to be identified, but an unknown component of the matrigel has been shown to increase its intrinsic activity (Sorby, Sandstrom et al. 2001).

1.7.1.3. Polymorphism

Analysis of the *PTPRJ* status in human tumors has shown that a LOH occurs in several tumors of breast, colon, thyroid and lung (Ruivenkamp, van Wezel et al. 2002; Ruivenkamp, Hermsen et al. 2003; Iuliano, Le Pera et al. 2004). The authors have suggested that the loss of one allele in the absence of any acquired mutation in the remaining allele can predispose to cancer, indicating *PTPRJ* haploinsufficiency as a transforming mechanism in humans. Different allelic variants of human *PTPRJ* have been reported and linked to different levels of cancer susceptibility. Usually these are polymorphisms affecting single aminoacids which are distributed in the extracellular fibronectin-like domains. One such polymorphism is Q276P. Tumor analyses of heterozygous patients with colon cancer indicated the preferential loss of the Q276 (resistance) allele and that the P276 allele is favored during cancer progression (Ruivenkamp, van Wezel et al. 2002). A similar analysis of thyroid tumors revealed an imbalance in another polymorphism, D872E (Iuliano, Le Pera et al. 2004). Recently, a similar study in breast cancer patients failed to identify imbalances on either of these polymorphisms but identified another single nucleotide polymorphism (SNP), in which the corresponding haplotype showed a weak but significant association with cancer progression (Lesueur, Pharoah et al. 2005).

1.7.1.4. Knock Out (KO) mice

An initial report showed that the homozygous knock out of DEP-1 is embryonically lethal. The authors of this study found structural abnormalities as early as embryonic day 8.25 (E8.25) and the embryos died at mid-gestation around E11.5. Death of the embryos was linked to vascularization defects as there was enhanced endothelial cell proliferation and enlargement of primitive vessels. However in this case, DEP-1 was replaced by a mutant allele $CD^{148\Delta CyGFP}$ (cDNA encoding DEP1-GFP fusion which lacks phosphatase activity) by in-frame replacement of cytoplasmic sequences with a green fluorescent protein (GFP) (Takahashi, Takahashi et al. 2003). In contrast, it has been shown recently that *Ptprj*-deficient mice are viable, fertile and show no gross anatomical alterations. Furthermore, neither changes in life span nor spontaneous tumor appearance were observed in *Ptprj* null mice indicating that DEP-1 may be dispensable for normal growth and development of mice (Trapasso, Drusco et al. 2006).

1.8 Aims

Density Enhanced Phosphatase-1 (DEP-1) is proposed as a tumor suppressor in a number of epithelial cancers, including that of breast, lung, thyroid and pancreatic carcinomas. It has also been mapped down to the colon cancer susceptibility locus (*Scc 1*) in mice. Nevertheless, its physiological role in colon epithelial cells is not known.

The aim of this thesis was to elucidate functional roles of DEP-1 in colon epithelial cells. To achieve this aim, two approaches should be used

- inducible re-expression of DEP-1 in a colon cancer cell line, which is devoid of endogenous DEP-1 protein.
- suppression of DEP-1 in a colon epithelial cell line normal endogenous levels using the si/shRNA approach.

Possible biological phenotypes related to transformation should be analyzed, including potential effects of changed DEP-1 levels on cell proliferation, migration, cell-matrix adhesion and apoptosis. Further, possible signaling mechanisms that might be altered due to re-expression or suppression of DEP-1 should be analyzed.

2. Materials & Methods

2.1. Materials

2.1.1. Chemicals

All general chemicals were purchased either from Carl Roth GmbH & Co (Karlsruhe, Germany) or Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany), and all molecular biology enzymes were purchased from New England Biolabs (Frankfurt, Germany) unless otherwise stated.

Compound / Substance	**Company**	**Cat. No.**
Epidermal Growth Factor (EGF)	Peprotech EC, Tebu bio, Frankfurt, Germany	100-15
Hepatocyte Growth Factor (HGF)	Peprotech EC, Tebu bio, Frankfurt, Germany	100-39
Lysophosphatidic acid (LPA)	Sigma Aldrich, Diesenhofen, Germany	L7260
Staurosporine (STS)	Alexis Biochemicals, Axxora, Grünberg, Germany.	Alx-380-014-C100
Anhydrotetracycline (Atc)	ACROS Organics, Fischer Scientific, Nidderav, Germany.	233131000
Immumount	Thermostandon, Pittsburgh, USA	9990402
DePeX	Serva Electrophoresis, Heidelberg, Germany	18243
Stromal Cell Derived Factor 1 alpha (SDF 1α)	Prospec-Tany Technogene Ltd, Israel	CHM 262B
Sphingosine 1 phosphate (S1P)	Calbiochem, Schwalbach, Germany	567727

2.1.2. Reagents and Buffers

Lysis buffers:

(A) 50 mM HEPES pH 7.5
150 mM NaCl
1.5 mM $MgCl_2$
1 mM EDTA
1% Triton X 100
1% Sodium desoxycholate
0.1% SDS
10% Glycerol

(B) 50 mM HEPES pH 7.5
150 mM NaCl
1 mM EDTA
2 mM EGTA
1% NP 40
10% Glycerol

(C) 50 mM Tris pH 7.5
150 mM NaCl
5 mM $MgCl_2$
1 mM EGTA
1% NP-40

Initial experiments were carried out with the lysis buffer "A". However later on it was replaced with the lysis buffer "B" without much discrepancy. For making the pull downs of active GTPases, lysis buffer "C" was used.

Added freshly before use:

1mM Dithiothreitol (DTT), Inhibitors (1 µg/ml Leupeptin, 1µg/ml Pepstatin A, 200 KIE/ml Aprotinin, 1 mM PMSF, 1 mM Benzamidine, 0.1 mg/ml Pefablock, 1mM sodium orthovanadate).

HNGT buffer (for washing Immunoprecipitates):

20 mM HEPES pH 7.5
150 mM NaCl
10% Glycerol
0.1% Triton X 100

NET-G-T:

50 mM Tris-HCl pH 7.5
150 mM NaCl
5 mM EDTA
0.04% Gelatine
0.05% Tween 20

6 x Sample buffer:

30% β-Mercaptoethanol
40% Glycerol
6% SDS
Bromophenol blue

Strip-buffer:

62.5 mM Tris-HCl pH 6.7
100 mM β-Mercaptoethanol
2% SDS

Transfer Buffer:

39 mM Glycine
48 mM Tris-HCl pH 9.0
20% Methanol
0.037% SDS

Coomassie staining:

2 g/l Coomassie R250
10% Acetic acid
50% Ethanol

Destaining:

10% Acetic acid
30% Ethanol

TSB (for the preparation of competent cells - *E.Coli* XL1Blue):

1 x Luria Broth medium
5 % DMSO
10 mM $MgCl_2$
10 mM $MgSO_4$
10 % PEG 4000

KCM buffer (for transformation):

0.5 M KCl
0.15 M $CaCl_2$
0.25 M $MgCl_2$

Luria Broth:

10 g - Bactotryptone
5 g - yeast extract
10 g - Sodium chloride
1.0 lit. - Water
pH adjusted to 7.0 – 7.4

BBS buffer (for calcium phosphate mediated transfections):

50 mM BES
280 mM NaCl
1.5 mM $Na_2HPO_4.2H_2O$
pH adjusted to 6.96.

pNpp buffer (for phosphatase assay):

100 mM HEPES (pH 7.4)
150 mM NaCl
1 mM EDTA
5 mM DTT (freshly added)

MACS buffer:

0.5 % BSA, 2 mM EDTA in PBS (pH 7.2). Degassed before use.

TBN 150:

25 mM Tris pH 7.5
150 mM NaCl
10 mM β-mercaptoethanol

Inhibitors (freshly added before use) - final concentrations:

10 mM β-Glycerol phosphate, 1 µg/ml Leupeptin, 1µg/ml Pepstatin A, 200 KIE/ml Aprotinin, 1 mM PMSF, 0.1 mg/ml Pefablock, 2 mM EGTA, 0.2mM EDTA

EQM buffer (equilibration buffer):

50 mM Tris-Cl pH 8.0
100 mM NaCl
10 mM DTT (freshly added)

EQL buffer (elution buffer):

EQM buffer + 10 mM Glutathione

Dialysis buffer:

100 mM NaCl
20 mM $NaH_2PO_4 \cdot H_2O$
80 mM $Na_2HPO_4 \cdot 2H_2O$
pH 7.5

2.1.3. Antibodies

Primary antibodies

Antigen	Type of Antibody	Dilution used in immuno-blotting	Company	Cat. No.
anti-β-actin	Mouse monoclonal	1:5000	Sigma Aldrich, Deisenhofen, Germany	A5441
anti-pan Akt	Rabbit polyclonal	1:2000	Cell Signaling, NEB, Frankfurt, Germany.	9272
anti-phospho Akt (ser^{473})	Rabbit monoclonal	1:2000	Cell Signaling, NEB, Frankfurt, Germany.	4058
anti-DEP-1 (143.41)	Mouse monoclonal	1:5000	Biosource Europe, Solingen, Germany.	AHT 4802
anti-pan Erk	Mouse monoclonal	1:2000	BD Biosciences, Heidelberg, Germany	610123

anti-phospho-p44/42 Erk [$pT^{202}Y^{204}$]	Mouse monoclonal	1:2000	Cell Signaling, NEB, Frankfurt, Germany	9106
anti-FAK	Rabbit polyclonal	1:1000	Cell Signaling, NEB, Frankfurt, Germany	3285
anti-FAK [pY^{397}]	Rabbit polyclonal	1:1000	Biosource Europe, Solingen, Germany	44-631
anti-FAK [pY^{407}]	Rabbit polyclonal	1:1000	Biosource Europe, Solingen, Germany	44-631
anti-FAK [pY^{576}]	Rabbit polyclonal	1:1000	Biosource Europe, Solingen, Germany	44-631
anti-FAK [pY^{577}]	Rabbit polyclonal	1:1000	Biosource Europe, Solingen, Germany	44-631
anti-FAK [pY^{861}]	Rabbit polyclonal	1:1000	Biosource Europe, Solingen, Germany	44-631
anti-phospho FAK [pY^{925}]	Rabbit polyclonal	1:1000	Cell Signaling, NEB, Frankfurt, Germany	3284
anti-Met (C-28)	Rabbit polyclonal	1:200	Santa Cruz Biotechnology, Heidelberg, Germany	sc-161
anti-phospho Met [$pYpYpY^{1230/1234/1235}$]	Rabbit polyclonal	1:1000	Biosource Europe, Solingen, Germany	44-888G
anti-PARP	Rabbit polyclonal	1:1000	Cell Signaling, NEB, Frankfurt, Germany	9542
anti-paxillin	Rabbit polyclonal	1:1000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-5574
anti-phospho paxillin [pY^{118}]	Rabbit polyclonal	1:1000	Cell Signaling, NEB, Frankfurt, Germany	2541
anti-PLC γ1	Rabbit polyclonal	1:2000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-426
anti-phospho PLC γ1 [pY^{783}]	Rabbit polyclonal	1:1000	Cell Signaling, NEB, Frankfurt, Germany	2821
anti-phospho tyrosine [4G10]	Mouse monoclonal	1:5000	Home made	
anti-Rac-1	Mouse monoclonal	1:1000	BD Biosciences, Heidelberg, Germany	610650
anti-Rho	Mouse monoclonal	1:1000	BD Biosciences, Heidelberg, Germany	R-73920
anti-pan Src	Rabbit polyclonal	1:2000	Biosource Europe, Solingen, Germany	44-656G
anti-c-Src (SRC 2)	Rabbit polyclonal	1:2000	Santa Cruz Biotechnology, Heidelberg, Germany	sc - 18
anti-phospho Src [pY^{529}]	Rabbit polyclonal	1:1000	Cell Signaling, NEB, Frankfurt, Germany	2105

anti-phospho Src [pY^{418}]	Rabbit polyclonal	1:2000	Biosource Europe, Solingen, Germany	44-660
anti-vinculin	Mouse monoclonal	1:5000	Biozol Diagnostica Vertrieb GmbH, Eching, Germany	BZL03106

All primary antibodies were either incubated with the immunoblots overnight at 4°C or at RT for one hour except for the anti-DEP-1 blot which was always performed overnight at 4°C.

Secondary antibodies / Modified antibodies

Antigen	**Source & Labelled / coupled with**	**Dilution used in immuno-blotting or labelling**	**Company**	**Cat. No.**
anti-mouse IgG	Goat / Peroxidase	1:10,000	KPL, Medac, Wedel, Germany	074-1806
anti-rabbit IgG	Goat / Peroxidase	1:10,000	KPL, Medac, Wedel, Germany	074-1506
anti-mouse IgG	microbeads	1:5 (20 µl / 10^7 cells)	Miltenyi Biotech, Bergisch Gladbach, Germany	130-048-401
anti-mouse IgG	Cy 3	1:600	Jackson Immunoresearch, Dianova, Hamburg, Germany	115-165-003
anti-rabbit IgG	TRITC	1:600	Jackson Immunoresearch, Dianova, Hamburg, Germany	111-025-003
anti-mouse IgG	DTAF	1:600	Jackson Immunoresearch, Dianova, Hamburg, Germany	115-015-072
anti-rabbit IgG	DTAF	1:600	Jackson Immunoresearch, Dianova, Hamburg, Germany.	111-015-144
anti-DEP-1	Mouse / FITC labelled	1:500	Santa Cruz Biotechnology, Heidelberg, Germany	sc-21761-FITC
Phalloidin	TRITC	1 µM	Sigma Aldrich, Deisenhofen, Germany	P1951

2.1.4. Cell Lines and Media

COGA-1, COGA-2, COGA-3 and COGA-12 cell lines were kindly provided by Dr. L.A. Huber (Research Institute of Molecular Pathology, Vienna, Austria). LT 97, a human colon adenoma cell line, was a kind gift from Prof. Brigitte Marian (Institute for Cancer Research, University of Vienna, Austria). All other cell lines were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH, Braunschweig, Germany). Unless otherwise stated, all cell culture vessels (flasks, dishes and freezing tubes) were purchased from Greiner bio-one (Cellstar, Frickenhausen, Germany) whereas all cell culture media (RPMI, DMEM and Trypsin) were purchased from PAA Laboratories, (Cölbe, Germany). Fetal Bovine Serum (FBS) was purchased from Biochrom, Berlin, Germany.

2.1.5. Plasmids and Primers

The plasmids pNRTIS 33 (DEP-1 WT) and pNRTIS 33 (DEP-1 C1239S) were kindly provided by Enrico Jandt (Jandt, Denner et al. 2003). The vector pNRTIS 21 was provided by Tencho Tenev (Tenev, Bohmer et al. 2000). The plasmid pSUPER.retro.puro was purchased from Oligoengine. The necessary oligonucleotides for shRNA constructs were purchased from Jena Biosciences, Jena, Germany.

441 (s)	5'-gat ccc c **GGGCCAGATCCTGTGCGCA** ttc aag aga **TGCGCACAGGATCTGGCCC**ttt ttg gaa a-3'
441 (as)	5'-agc ttt tcc aaa aa **GGGCCAGATCCTGTGCGCA** tct ctt gaa **TGCGCACAGGATCTGGCCC** ggg-3'
1151 (s)	5'-gat ccc c **TACAACATCAACCCGTATC** ttc aag aga **GATACGGGTTGATGTTGTA** ttt ttg gaa a-3'
1151 (as)	5'-agc ttt tcc aaa aa **TACAACATCAACCCGTATC** tct ctt gaa **GATACGGGTTGATGTTGTA** ggg-3'
1524 (s)	5'-gat ccc c **CGAGTCGTCATCTAACTAT** ttc aag aga **ATAGTTAGATGACGACTCG** ttt ttg gaa a-3'
1524 (as)	5'-agc ttt tcc aaa aa **CGAGTCGTCATCTAACTAT** tct ctt gaa **ATAGTTAGATGACGACTCG** ggg-3'

2348 (s)	5'-gat ccc c **ATTCCAGCAACGCAACACA** ttc aag aga **TGTGTTGCGTTGCTGGAAT** ttt ttg gaa a-3'
2348 (as)	5'-agc ttt tcc aaa aa **ATTCCAGCAACGCAACACA** tct ctt gaa **TGTGTTGCGTTGCTGGAAT** ggg-3'
Non-targeting control (s)	5'-gat ccc c **AATTCTCCGAACGTGTCACGT** ttc aag aga **ACGTGACACGTTCGGAGAATT** ttt ttg gaa a-3'
Non-targeting control (as)	5'-agc ttt tcc aaa aa **AATTCTCCGAACGTGTCACGT** tct ctt gaa **ACGTGACACGTTCGGAGAATT** ggg-3'

2.2. Methods

2.2.1. Cell Culture

All the cells were cultured either in RPMI or in DMEM, supplemented with 10% FBS. For routine passage, they were cultured in 75 cm^2 flasks in an incubator (Thermo Electron Corporation, Langenselbold, Germany) maintained at 37^oC, 5% CO_2 and 95% humidity. The cells were allowed to become 80% confluent before they were split. For splitting, the media was removed and the cell layer was washed twice with a solution of Trypsin-EDTA. Usually, a splitting ratio of 1:10 or 1:20 was used. Freezings: Cells were frozen with the usual culture medium containing 10% DMSO. They were aliquoted into special freezing tubes (Cellstar, Frickenhausen, Germany) and allowed to freeze in a polystyrene container at -80^oC. Then, they were stored in liquid nitrogen. For thawing, the cells were removed from the liquid nitrogen, quickly thawn at 37^oC and spun down (to remove DMSO). The cell pellet was then mixed with fresh growth medium and new cultures were initiated.

The adenoma cells, LT 97 (Richter, Jurek et al. 2002) was cultivated in a specialized mixture of media containing 400 ml of MCDB Basal Medium (without glutamine) with $NaHCO_3$ (Biochrom, Berlin, Germany) and 100 ml of L-15 Leibovitz medium (Biochrom, Berlin, Germany). A number of supplements were added including 2 mM Glutamine, 2% FBS, Insulin-Transferrin-Selenium-mixture (Gibco, Invitrogen,

Karlsruhe, Germany, Cat. No. 41400-045), 0.2 nM Triiodothyronine, 1 µg/ml Hydrocortisone, 30 ng/ml Epidermal Growth Factor (Calbiochem, Schwalbach, Germany). For splitting, a solution of PBS with 5 mM EDTA was used and splitting was carried out as follows: after removal of media, the cells were incubated with PBS/EDTA for 5-7 min at 37°C. The PBS/EDTA was then removed and cells were incubated for another 3-4 min at 37°C. The cells were now readily detached with a bit of mechanical agitation. After mixing with growth media, the suspension was used for further passaging or seeded for experiments.

2.2.2. Cloning

The cDNAs corresponding to DEP-1 WT or CS were sub-cloned from pNRTIS 33 into pNRTIS 21. Briefly, the vector pNRTIS 21 was first digested with EcoRI followed by dephosphorylation of the ends with Calf Intestinal Phosphatase (CIP, Sigma-Aldrich, Deisenhofen, Germany). The cDNA insert was excised from parental plasmids also with EcoRI. Ligation was performed with dephosphorylated vector and insert (ratio of 4:1) overnight at 12°C. The ligation mixture was transformed into competent XL1Blue bacteria and positive clones were screened by making mini preparations of plasmids (Qiagen, Hilden, Germany).

Digestion with restriction enzymes

pNRTIS 21 (conc. 1 µg/µl)	5 µl
EcoRI (20 U/µl)	2 µl
Buffer (10 x)	5 µl
Water	38 µl
Total	50 µl

The reaction mixture was incubated at 37°C for two hours. The enzyme was inactivated at 65°C with 20 min incubation time.

Dephosphorylation of vector

30 µl of the digested vector was mixed with 3 µl of 10 x CIP buffer and 1 µl CIP. It was carried out for two hours at 37°C after which it was inactivated with 0.5% SDS and 5 mM EDTA at 65°C for 30 min. DNA was then precipitated with 0.3 M of sodium acetate, 1 µl of Linear Acrylamide [LAA was kindly provided by Dr. Lucho Karagyozov and prepared (Gaillard and Strauss 1990) with a stock conc. - 10µg/µl] mixed with an equal volume of

isopropanol. It was spun down and washed with 70% ethanol and the pellet was resuspended in TE buffer.

Digestion with Restriction enzymes to excise insert

The plasmids harboring either DEP-1 WT or CS in pNRTIS 33 were also digested as described above with EcoRI.

Ligation

40 ng of dephosphorylated vector was mixed with 10 ng of insert in a volume of 20 µl. 1 µl of DNA ligase (3U/µl) and 2 µl of 10 x ligase buffers with 10 mM ATP were then added and incubated overnight at 12^{o}C.

Transformation

80 µl of competent *E.Coli* cells (XL1Blue) was mixed with 20 µl of 5 x KCM buffer along with 10 µl of ligation mixture. The sample was kept on ice for 15 min after which it was heat-shocked at 42^{o}C for 2 min and brought back to ice. 1.0 ml of Luria Broth medium (without antibiotics) was added and incubated at 37^{o}C. Half of the transformation mixture was used for plating on Luria Broth plates containing ampicillin (100 µg/ml) and the resistant colonies were allowed to grow overnight at 37^{o}C.

Minipreparations of plasmids

The resistant colonies were inoculated in 3.0 ml Luria Broth medium each, allowed to grow overnight and plasmids were extracted using a Qiaprep Spin Miniprep Kit (Qiagen, Hilden, Germany) as described by the manufacturer.

Checking the clones

The clones were analysed to check whether they have the insert in the right orientation by performing a double digestion with Hind III (cuts at 3' end of the insert) and EcoRV (cuts in the vector pNRTIS 21). The digested fragments were electrophoresed on 1% agarose gels and positive clones were detected.

2.2.3. Transient Transfections – HEK 293 cells

Transient transfections to analyse expression were carried out in HEK 293 cells using calcium phosphate. In brief, HEK 293 cells were routinely cultured in DMEM/F-12 medium supplemented with 10% FBS. For transfections, cells were trypsinized and 3.5 x 10^5 cells/2.0 ml/well were seeded in 6 well plates. A specialized mixture of transfection medium containing one part of DMEM/F-12 and two parts of DMEM, both

supplemented with 10% FBS were used for this purpose. 24 hours later, DNA precipitates were made by mixing 96 µl of pre-mix (8.6 ml of H_2O and 1 ml of 2.5 M $CaCl_2$), 4 µg of plasmid DNA and 100 µl of BBS buffer and incubated for 20 min at RT. The precipitates were then added in drops to the cells, gently shaken and incubated at 3% CO_2 incubator overnight. The next day, fresh media was replaced and the cells were put back to the normal 5% CO_2 incubator. It was incubated for additional 24 hours after which the cells were lysed.

2.2.4. Generation of Stable Cell Lines

SW 480 cells were transfected using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany). The ratio between DNA and Lipofectamine 2000 used was 1:4. The plasmids pNRTIS 21 with either DEP-1 WT or DEP-1 CS inserts were first linearized with Bst Z171 in order to ease integration into the genomic DNA. Two days after transfection, the cells were selected with the antibiotic G418 (PAA Laboratories, Cölbe, Germany) at a concentration of 200 µg/ml. Selection was carried out for a period of two weeks. The resistant pools were then trypsinized and diluted in a 96 well plate (1 cell/100µl/well) for clonal selection. All these steps were carried out in the presence of 150 ng/ml of Anhydrotetracycline (Sigma Aldrich, Deisenhofen, Germany) in order to suppress DEP-1 expression. Those wells that harbored single clones (arising from single cells) were then trypsinized and further expanded and analysed for their expression in western blots.

2.2.5. Cell Sorting - MACS

Magnetic Assisted Cell Sorting (Milltenyi Biotech, Bergisch Gladbach, Germany) was performed to enrich the DEP-1 expressing cells. At first, the cells were cultured without Atc for four days completely relieving the suppression. The cells were then mildly trypsinized by quickly washing the monolayer of cells twice with a solution of Trypsin / EDTA followed by very short incubation (usually around 1 min) at 37^{o}C. The cells were readily detached with a bit of mechanical agitation and were suspended in 5.0 ml of fresh media with serum. The serum will inhibit the trypsin and the cells were then pelleted by centrifuging at 1,000 rpm for 3 min. The cells were blocked by suspending the pellet in PBS containing 10% FBS and incubating them at RT for 10 min. Anti-DEP-1 antibody (2µg/500µl) in MACS buffer was added and incubated for additional 30 min at RT

followed by washing thrice with MACS buffer. The secondary antibody coupled to magnetic beads was then added (20µl antibody + 80 µl MACS buffer) and incubated for 15 min at 4^{o}C. The cells were washed thrice again in the same buffer (5 min each). Meanwhile, the MACS column is placed in the magnetic field of MACS separator and is equilibrated by washing thrice with 500 µl of MACS buffer. The samples were then loaded onto the column. Unbound cells were removed by washing the column thrice with 500 µl of the same buffer. The column was then separated from the magnetic field and the bound cells were eluted with the plunger provided.

2.2.6. Phosphatase Assay - pNpp assay

Para-nitrophenylphosphate (pNpp) was used as the substrate for this assay. The assay is based on the cleavage of the phosphate group from pNpp (due to the enzyme activity of the phosphatase) turning into nitrophenol (yellow colour substance). This can be quantified by measuring the absorbance at 405 nm. The assay was started by mixing of pNpp substrate (10 mM), buffer and sample to a total volume of 50 µl. The mixture was incubated for 30 min at RT. The reaction was terminated by addition of 100 µl of 1 M NaOH after which absorbance was measured at 405 nm.

2.2.7. Proliferation Assay

2.2.7.1. Cell counter

100,000 cells/2.0ml/well were seeded in a 6 well plate. On alternative days, they were trypsinized and counted in a Casy-1 cell counter (Schärfe Systems, Reutlingen, Germany). Assays were always performed in triplicates.

2.2.7.2. DAPI assay

The assay is based on the ability of 4', 6-Diamidino-2-phenylindole (DAPI) to bind to DNA which will then fluoresce. The assay was usually carried out in 96 well plates. 8,000 cells/100 µl/well were seeded and were treated wherever necessary. On the respective days, cells were first fixed with methanol (100 µl/well for 5 min). Following the removal of methanol, the plate was just left open for 15 min at RT in order to remove the residual moisture. A 20 µM solution of DAPI (100 µl/well) in PBS was then added, followed by incubation at 37^{o}C for at least 30 min (formation of DNA-DAPI complexes).

The plate was then measured in a fluorescent plate reader (TECAN Spectrafluor GmbH, Crailsheim, Germany) using the following parameters: Ex/Em - 360/465, Gain - 60, Reading mode - bottom and multiple readings per well.

2.2.8. Migration Assay

2.2.8.1. Wound healing assay

A sterile 6 well plate was taken and the bottom side of each well was marked to position the wound and five random fields were also marked to aid in quantification towards the end of the assay. 200,000 cells/2.0ml/well were seeded in 6 well plates, allowed to grow to confluence, and starved overnight. The cell layer was wounded by scratching with a sterile 1.0 ml pipette tip. The wound was created in such a way that it coincided with the marked place at the bottom. Detached cells were removed by washing three times with serum-free medium. Medium containing 10% fetal bovine serum (or other motogens) was added. The plates were now examined under a microscope at all the five marked places using an ocular with grids and the positions were noted. It was incubated at 37^{o}C for 24 h. The plates were then examined exactly at the same positions by aligning the ocular with grids to the wound. The numbers of migrated cells at all these five fields were counted.

2.2.8.2. Transwell assay

The assay was carried out in a modified 12 well plate which contains an insert with a basal membrane of defined pore size of 8 µM (12 mm well chambers; Transwell Costar Corp., Cambridge, MA). The membrane was first coated with a 50 µg/ml collagen type I solution (Sigma-Aldrich, Deisenhofen, Germany, Cat. No. C9791) by incubating for one hour at 37^{o}C followed by washing thrice with PBS. Starved cells (50,000 cells/500 µl) were seeded on top of the collagen-coated membrane, and cells migrating to the bottom side (towards stimulus) were scored after 6 hours by staining with 0.5% crystal violet in 20% methanol for 5 min. followed by washing thrice with PBS. The cells on the top of the membrane (non-migrated) were removed using a cotton swab. The membrane was gently excised using a scalpel from the attached insert and mounted on a glass slide with DePeX mounting solution. The cells on the bottom side were then counted using a microscope. Five different fields randomly chosen were counted and quantified.

2.2.9. Adhesion Assay

Adhesion assays were carried out in two different ways. The first method is based on the rate at which the cells attach to the substratum, whereas the second one scores the rate at which the cells can detach. In the first method, 96 well plates were coated with 50 µg/ml collagen type I (Sigma-Aldrich, Deisenhofen, Germany, Cat. No. C9791) at 4°C overnight and then washed twice with PBS. Cells were suspended at a density of $3x10^5$ cells/ml in serum-free medium and kept in suspension for 30 min, after which 100 µl cell suspension was added per well. Adhesion was allowed for different time points and was stopped by removal of the medium with non-adhered cells. The adhered cells were then stained with 0.5% crystal violet in 20% methanol for 5 min. The wells were washed twice with PBS, and the dye was solubilized in 0.1 M sodium citrate (100 µl per well) and measured at 570 nm. In the second method, cells were plated in 24 well plates in quadruplicates at a density of $1x10^5$ cells/well. Cells were allowed to adhere by incubating them at 37°C for one day and then gently washed initially with 500 µl of Ca^{2+} and Mg^{2+} free PBS followed by incubating again with a solution of PBS or with a solution of Accutase (PAA Laboratories, Cölbe, Germany). Detached cells were removed by aspirating them along with the medium. The remaining attached cells were scored by fixing and staining with methanol and crystal violet as described above.

2.2.10. Immunofluorescence and Phalloidin Staining

Cells were plated on collagen-coated coverslips in 24 well plates (10,000 cells/ml/well) followed by washing with PBS. They were fixed with 4% paraformaldehyde in PBS (pH 7.0-7.4) by incubating for 10 min at RT followed by washing thrice with PBS. The cells were then permeabilized with 0.2% Triton X -100 in PBS at RT for 4 min followed by blocking with 1.5% BSA in PBS. Phalloidin or primary antibodies were dissolved at the desired concentrations (phalloidin - 1 µM, vinculin - 1:500 or phospho-paxillin - 1:100) and the coverslips were incubated either for one hour at RT or overnight at 4°C. They were then washed thrice with 1.5% BSA/PBS (20 min each). Corresponding Cy3 or TRITC labelled secondary antibodies with a dilution of 1:600 were then added and the coverslips incubated for one hour at RT, then washed thrice with PBS. The nuclei were stained with DAPI (2 µg/ml in PBS) by incubating the coverslips at RT for 5 min. The coverslips were finally washed thrice with PBS and then mounted on glass slides with

mounting solution (Immumount). The slides were dried overnight at RT (dark conditions) before examination under a microscope. Pictures were taken with a Zeiss AxioCam HRC digital camera system, mounted on a Zeiss Axioplan2 microscope, and the AxioVision 3.1 software. The following filter sets were used for examination of the slides.

Channel	Filter set numbers	Excitation	Beam splitter	Emission	Cat. No.
Red	Filter set 15	BP 546/12	FT 580	LP 590	488015-0000-000
Green	Filter set 9	BP 450-490	FT 510	LP 515	488009-0000-000
Blue	Filter set 1	BP 365/12	FT 395	LP 397	488001-0000-000

2.2.11. Apoptosis Assay

A Cell Death Detection ELISA kit (Roche Diagnostics, Mannheim, Germany, Cat. No. 1774425) was employed. The assay is based on a quantitative sandwich-enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones which allows determining the mono- and oligonucleosomal fraction in the sample. In brief, 10,000 cells/well in a 24 well plate (sometimes higher cell numbers were used but the lysates were diluted before analysis) were seeded and allowed to settle overnight. The next day, the cells were treated with different concentrations of staurosporine for 4 hours. They were then lysed in the lysis buffer (provided in the kit) at RT for 30 min and then centrifuged at 200 x g for 10 min. Cell lysates corresponding to 1 x 10^3 cells were transferred to the provided streptavidin coated microtitre plate. A mixture of biotin-labeled histone antibodies and peroxidase-conjugated anti DNA antibodies was added and the plates were incubated at RT for further two hours. The unbound antibodies were removed by washing thrice with the incubation buffer provided. The bound peroxidase was determined photometrically at 405 nm with ABTS as substrate. Since the assay is quite sensitive, it is essential to have equal inputs for all the samples under investigation. For this purpose, similar amounts of cells were seeded in parallel on the first day of the experiment. They were then lysed and protein amounts were estimated by performing either BCA assay (BCA Protein Assay Kit, Perbio Science, Bonn, Germany, Cat. No. 23235) as described by the manufacturer or by immunoblotting the lysates against vinculin antibody. The obtained data were then used to normalize the

data of the apoptosis ELISA. Apoptosis was also detected by monitoring the cleavage of poly (ADP-Ribose) polymerase (PARP-1), using an antibody that detects both the cleaved (89 kDa) and uncleaved (116 kDa) forms in immunoblotting.

2.2.12. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

A single step RT-PCR reaction was performed in order to detect the presence of DEP-1 mRNA in the cell populations under study. Briefly, 2 x 10^6 cells were seeded in 6.0 cm. dishes and allowed to settle overnight. Total RNA was isolated using RNeasy kit (Qiagen, Hilden, Germany). 1 µg of the total RNA was used to perform a single step RT-PCR using the One Step RT-PCR kit (Qiagen, Hilden, Germany, Cat. No. 210212) under the following conditions and the resulting products were analysed in 1% agarose gel electrophoresis.

Cycle 1 (1x): cDNA synthesis

Step 1:	50 ^{o}C	30 min.

Cycle 2 (1x): Initial Denaturation

Step 1:	95 ^{o}C	15 min.

Cycle 3 (35x): Amplification

Step 1:	95 ^{o}C	30 sec.
Step 2:	54 ^{o}C	60 sec.
Step 3:	72 ^{o}C	45 sec.

Cycle 4 (1x): Final Extension

Step 1:	72 ^{o}C	5 min.

Cycle 5 (1x): Pause

Step 1:	4 ^{o}C	infinite

Forward primer: 5'-CCTGGTTATTGTGACTGTGGAGG-3'

Reverse primer: 5'-TCTGGACCGAAAGTTTGACACG-3'

2.2.13. Cell lysis, Immunoprecipitation and Blotting

Cells were usually grown either in 6 well plates or in 6.0 cm dishes to 70% - 80% confluence. Wherever necessary, starvation was always performed overnight in serum free medium. Stimulations were usually done with 10% FBS (unless otherwise specified) for the indicated time periods. For preparing cell extracts, cells were washed with ice-

cold PBS, followed by lysing with lysis buffer (300 µl/well in a 6-well plate or 500 µl/6.0 cm. dish). The cells were then scraped off and centrifuged at 14,000 rpm at 4°C for 15 min. The supernatant was either subjected to immunoprecipitation (IP) or to western blotting. IP of DEP-1 was performed by mixing lysates with mouse monoclonal anti-DEP-1 antibody (0.5-1.0 µg/sample) and incubating them at 4°C for one hour in a rotary wheel. 20 µl of protein A-sepharose bead suspension per sample was then added and incubated for another hour. The beads were then washed thrice with HNGT buffer, boiled in 30 µl of 2 x sample buffer and finally subjected to electrophoresis.

For western blotting, usually 10% of cell lysates were subjected to polyacrylamide gel electrophoresis and thus separated. The percentage of the gel casted was dependent on the size of the protein of interest. The gel was then transferred to PVDF membrane (Millipore, Eschborn, Germany) by a Semidry Transfer Cell System (Millipore). The membranes were blocked with NET-G-T solution, either for one hour at RT or at 4°C overnight. They were then probed with primary antibody (specified dilution) either for one hour at RT or overnight at 4°C followed by washing thrice with NET-G-T. The appropriate secondary antibody (1:10,000) coupled to horse-radish peroxidase was then added and incubated at RT for another hour. They were finally washed thrice with NET-G-T and the signals were detected with chemiluminescent reagents [Western lightening, Chemiluminiscent Reagent Plus (Cat. No. NEL104, NEL 105) or SuperSignal West Dura Extended Duration Substrate (Cat. No. 34075), Perkin Elmer, Rodgau Jügesheim, Germany] by exposing them to X-ray films (CL-XPosure Film, Perbio Science, Bonn, Germany, Cat. No. 34090). To reprobe the same membrane with another antibody, the blots were first stripped by incubating them with stripping buffer at 56°C for 20-30 min followed by three washes with NET-G-T and detection with another antibody as described.

2.2.14. Density Dependent Upregulation

In order to determine the density dependent upregulation, HT 29 cells were seeded at different densities either in 6.0 cm or in 10.0 cm dishes (cell numbers are given in the table below). The following day, the cells were lysed and IP of DEP-1 was performed as

described before. Loading controls were performed by immunoblotting of lysate samples with anti-vinculin antibodies.

Confluence (x 10^4 cells/cm^2)	**Diameter of the dish**	**No. of cells seeded**
1	10 cm	6 x 10^5
2.5	10 cm	15 x 10^5
5	10 cm	30 x 10^5
10	6 cm	20 x 10^5
25	6 cm	50 x 10^5
75	6 cm	150 x 10^5

2.2.15. GTPase Pull Downs

The binding domains of small GTPase effectors were cloned in pGEX vectors (Pharmacia, Amersham Biosciences, Freiburg, Germany) and were kindly provided by Dr. Ignacio Rubio. The pGEX vectors offer several advantages including the presence of a tac promoter which allows chemically inducible, high-level expression. Moreover, the target proteins can be expressed as a fusion protein with Glutathione-S-Transferase (GST) tag which allows easy purification through the GST purification module. The cDNA for Rhotekin (the part that binds to active Rho) was used to pull down active Rho. Similarly the Rac binding domain of PAK (Rac effector) is used to pull down active fractions of Rac.

2.2.15.1. Preparation of the binding domain

The pGEX plasmids harboring the cDNA for the binding domains were transformed into the *E.coli* strains BL21-DE3. In brief, 50 ml of overnight culture was inoculated into 500 ml Luria Broth containing ampicillin. When the culture had reached an OD_{600nm} of 0.7, it was induced with 0.1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) and incubated at 37^oC for additional 5 hours. The bacterial cells were then pelleted and resuspended in approximately 8.0 ml of TBN150 with protease inhibitors. The bacteria were lysed by freezing and thawing thrice and by incubation with 5 mg of lysozyme for 20 min at RT. 280 µl of 500 mM EDTA and 350 µl of 10% Triton X-100 were then added and incubated at RT for further 20 min, after which it was centrifuged at 30,000

rpm at 4°C for 30 min. The supernatant was mixed with 1.0 ml of Glutathione Sepharose (GSH) beads pre-equilibrated with TBN 150 and incubated in a rotary wheel at 4°C for one hour. Washing was initially done twice with 10.0 ml of TBN 150 containing 1% Triton X-100 followed by washing twice again with EQM buffer. The binding domains were finally eluted from the bound GSH beads with 10.0 ml of EQL buffer. The eluate was finally concentrated (Amicon Ultra, Millipore, Eschborn, Germany, Cat. No. UFC801008), dialysed against PBS overnight and frozen in aliquots at -80°C.

2.2.15.2. Pull downs

In brief, 500,000 cells/5.0ml/6.0 cm dishes were seeded and allowed to grow for 2-3 days. Starvation was done overnight after which the cells were stimulated for the indicated time periods. Lysis buffer "C" was used to lyse the cells for performing pull down of active Rho / Rac GTPases. Generally, cells were lysed in 1.0 ml of ice cold lysis buffer containing the corresponding binding domains (25 µg/ml) and GDP (100 µM) and centrifuged at 14,000 rpm at 4°C for 15 min. The supernatants were then mixed with 40 µl of GSH-sepharose beads pre-equilibrated with lysis buffer. It was incubated in a rotary wheel for 30 min at 4°C. The beads were washed twice with the same lysis buffer and eluted with 30 µl of 2 x gel loading buffer as described for immunoprecipitation.

2.2.16. Half-life Determination

Pulse chase method: HEK 293 cells were transiently transfected with cDNA encoding DEP-1 WT using calcium phosphate. Cells were washed twice with PBS after which they were starved. Starvation was carried out using a special DMEM medium devoid of L-glutamine, sodium pyruvate, L-methionine and L-cysteine (Gibco, Invitrogen, Karlsruhe, Germany, Cat. No. 21013-024). This medium was supplemented with 1 mM pyruvate and 2 mM glutamate and thus cells were starved only for cysteine and methionine. Starvation was carried out at 37°C for one hour. The cells were then pulsed for 3 hours with 200 µCi/ml of ^{35}S (Redivue PRO-MIX Cell Labelling Mix, Hartmann Analytik, Braunschweig, Germany, Cat. No. AGQ0080) and contains both L-[^{35}S] methionine and L-[^{35}S] cysteine with a specific activity of 37 TBq/mmol (1000 Ci/mmol). Three hours later, the radioactive medium was collected, and the cells were washed with PBS followed by replacing with fresh, non-radioactive medium (referred to as "chase"). For

chasing, the same media supplemented not only with 1 mM pyruvate and 2 mM glutamate but also supplemented with 0.2 mM cysteine and 0.1 mM methionine was used. Thereafter, at the specified time points, the cells were washed with PBS and lysed in 300 µl of ice-cold lysis buffer. IP was performed with anti-DEP-1 antibody and the immunoprecipitates were separated with 7.5% polyacrylamide gel. The gel was first stained with Coomassie followed by soaking in Intensify A and then Intensify B (Du Pont, Perkin Elmer, Rodgau Jügesheim, Germany) for 45 min each. The gel was finally dried and the resulting fluorographic signals were detected by exposure to X-ray film with an intensifying screen for a week at -80^{o}C. The resulting images were scanned and densitometric analyses were performed with NIH image software.

2.2.17. shRNA Cloning

The pSuper.retro.puro vector (Oligoengine, Seattle, USA) was double digested with Hind III and Bgl II and the ends were dephosphorylated as described before and purified through a low melting agarose gel (S.N.A.P Gel Purification Kit, Invitrogen, Karlsruhe, Germany, Cat. No. K1999-25). The 64-mer oligos were annealed as described by the manufacturer. In brief, the oligos were dissolved in water to a concentration of 3 mg/ml. An annealing mixture containing 1 µl of each oligo, 10 µl of 5 x annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4 and 2 mM magnesium acetate) and 38 µl of water were prepared. It was incubated at 95^{o}C for 4 min and cooled down slowly to 25^{o}C. The annealed oligos were then precipitated with sodium acetate and isopropanol, and dissolved in TE buffer. They were phosphorylated with polynucleotide kinase (PNK) (Roche Diagnostics, Mannheim, Germany) in the presence of PNK buffer containing 1 mM ATP by incubating them at 37^{o}C for 30 min. The enzyme was then heat inactivated by incubation at 70^{o}C for 10 min. The annealed, phosphorylated oligos were then ligated with digested vector using DNA ligase as described before except that ligation was carried out for one hour at RT. The ligated mixture was transformed into competent *E.Coli* XL1Blue cells. Plasmids were extracted and double digested with EcoRI and Hind III to check for the inserts.

2.2.18. Retroviral Infection

Phoenix-Ampho cell lines were used for the generation of helper free amphotropic retroviruses. Phoenix is based on the 293T cell line which is engineered to harbor three essential retroviral genes - group-specific antigen (gag), polymerase (pol) and envelope (env). Briefly, phoenix cells were transfected with the shRNA constructs (construct 441 and the non-targeting control) using calcium phosphate. After transfecting, the cells were moved to a Safety Level 2 (S2) incubator. In parallel, the target cells (HT 29) were also seeded for infection (2 x 10^5 cells/well/24 well plate).) 24 h after the transfected cells became confluent, viruses were collected (along with the medium). The medium was either centrifuged or filtered (0.45 µM) in order to remove any swimming cells. The resulting viral supernatants were now used to infect the target cells (HT 29) along with polybrene (10 µg/ml). Medium was changed after 4-6 h of incubation as polybrene is toxic for the cells. The next day, a similar second round of infection was performed to attain the maximum efficiency of infection. The cells were then brought back to Safety Level 1 (S1) incubator and two days later, they were selected with the antibiotic puromycin (2 µg/ml). The resistant cell pools were further expanded and checked for their suppression.

2.2.19. Phospho RTK Array

This technique allows to simultaneously identifying the relative levels of phosphorylation of 42 different RTKs. An array of antibodies to 42 RTKs, spotted on a nitrocellulose membrane was purchased from R & D Laboratories (Wiesbaden, Germany, Cat. No. ARY001). Probing of the membranes was done according to the protocol of the manufacturer. In brief, sub-confluent dishes of cells were starved overnight followed by stimulation with FBS for 15 min. The cells were lysed in lysis buffer (1% NP-40, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 200 KIE/ml Aprotinin and 10 µg/ml Leupeptin) and placed in a rotary wheel at 4^{o}C for 30 min. The mixture was then centrifuged, and the supernatant was collected. Equal amounts of supernatant (around 70% of the cell lysates) were applied on blocked membranes and incubated overnight at 4^{o}C. The membranes were washed thrice (10 min each) in the wash buffer provided. Anti phospho-tyrosine antibody (dilution of 1:2000) coupled to HRP was added, and incubated for additional two hours. The arrays

were finally washed thrice followed by exposing them to chemiluminiscent reagents and the resulting signals were scored on films.

2.2.20. Treatment with Different Nutrient Components

A freeze dried polyphenol extract from a mixture of table apples (designated AE) was kindly provided by Dr. F. Will (Research Institute Geisenheim, Germany). For cell treatments, the lyophilisate was freshly dissolved in medium and sterile filtered. A green tea extract (GT) was kindly provided by Kampffmeyer Food Service GmbH (Hamburg, Germany). Subconfluent cultures of colon cells (HT 29, CaCo2 and LT 97) were either left untreated or were treated for 72 h with 2 mM sodium butyrate, apple polyphenol extract (AE) at a concentration equivalent to 25 µM phloridzin or green tea (GT) at a concentration equivalent to 20 µM epigallocatechine-3-gallate. The cells were lysed and IP was performed with anti-DEP-1 antibody as described earlier. Lysate aliquots were analysed for actin levels as input control. In addition, LT 97 cells were also treated with different concentrations of apple polyphenol extract (concentrations equivalent to 0, 1, 2.5, 15 or 25 µM phloridzin) for 72 h and DEP-1 expression was again determined by immunoprecipitation.

2.2.21. Real Time PCR

This technique allows monitoring the relative mRNA levels of genes. Subconfluent LT 97 cells were treated as described above and total RNA was extracted with RNeasy kit (Qiagen, Hilden, Germany). The RNA concentration was measured using spectrophotometer (ND-1000, Nanodrop, Peqlab Biotechnology, Erlangen, Germany). 2 µg of RNA was used to synthesize cDNA using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Karlsruhe, Germany, Cat. No. 11904-018) as instructed by the manufacturer. The cDNA was subjected to real-time-polymerase chain reaction using an iCycler and iQ SYBR Green Supermix (BioRad, Munich, Germany, Cat. No. 170-8884) detection as instructed by the manufacturer using the following conditions. Values were normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) - mRNA detected in the same samples.

Conditions for Real Time PCR:

Cycle 1:	(1x)	Initial denaturation		
	Step 1:		95.0ºC	2 min.
Cycle 2:	(40x)	Amplification		
	Step 1:		95.0ºC	35 sec.
	Step 2:		60.0ºC	30 sec.
	Step 3:		72.0ºC	40 sec.
Cycle 3:	(1x)	Final Extension		
	Step 1:		72.0ºC	10 min.
Cycle 4:	(80x)	Denaturation (melting curve analysis)		
	Step 1:		55.0ºC	10 sec.
Cycle 5:	(1x)	Pause		
	Step 1:		4.0ºC	infinite

For human DEP-1: Forward primer: 5'-TCGTTCGTGACTACATGAAGCA-3'
Reverse primer: 5'-CCCCAGCACTGCAATGC-3'

For human GAPDH: Forward primer: 5'-CCCATGTTCGTCATGGGTGT-3'
Reverse primer: 5'-TGGTCATGAGTCCTCCACGATA-3'

Animal experiments were performed elsewhere and the RNA samples were kindly provided by Dr. Stefan W. Barth (Karlsruhe, Germany). Briefly, rats were fed with apple juice. Water was used as the control. One group of animals was also injected with the carcinogen DMH or sodium chloride as the control. The mucosa was scraped, and total RNA was isolated from these tissues. cDNA synthesis and Real Time PCR were done as before, except that rat-specific primers were used. Primer sequences for the rat genes and conditions for Real Time PCR were kindly provided by Dr. Kai Kappert, Cancer Centre, Karolinska, Stockholm, Sweden. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) was used to normalize the values instead of GAPDH.

For rat DEP-1: Forward primer: 5'-TTTTGAAGCCAGCCATGGA-3'
Reverse primer: 5'-GTGGAGGTTGGCCAGCT-3'

For rat HPRT: Forward primer: 5'-CTCATGGACTGATTATGGACAGGAC-3'
Reverse primer: 5'-GCAGGTCAGCAAAGAACTTATAGCC-3'

3. Results

3.1. Characterisation of Density Enhanced Phosphatase-1 (DEP-1)

3.1.1. Analysis of Endogenous DEP-1 Levels in Colon Epithelial Cell Lines

Genetic analysis has shown allelic loss of the DEP-1 encoding gene *PTPRJ* in cases of human colon cancer. However, little is known about DEP-1 protein expression in colon cancer cells. We therefore analysed a panel of colon epithelial cell lines (including one adenoma cell line, LT 97) for DEP-1 protein expression. Cells from one confluent 75 cm^2 flask each were subjected to immunoprecipitation (IP) with anti DEP-1 antibody, followed by immunoblotting. As shown in Fig. 3.1a, expression in the analysed cell lines varies from relatively high levels in LT 97 adenoma cells and HT 29, DLD-1, HCT-16, COGA-1 and COGA-12 colon carcinoma cells, to relatively low levels in LOVO, Colo-320, COGA-2 and COGA-3 cells, and no detectable DEP-1 protein expression in SW 480 cells. Interestingly, the relatively abundant DEP-1 expression in the adenoma cell line LT 97 may reflect its resemblance to normal tissue. Overall, DEP-1 expression is rather heterogeneous, and low levels of DEP-1 are not a uniform feature of colon cancer cells.

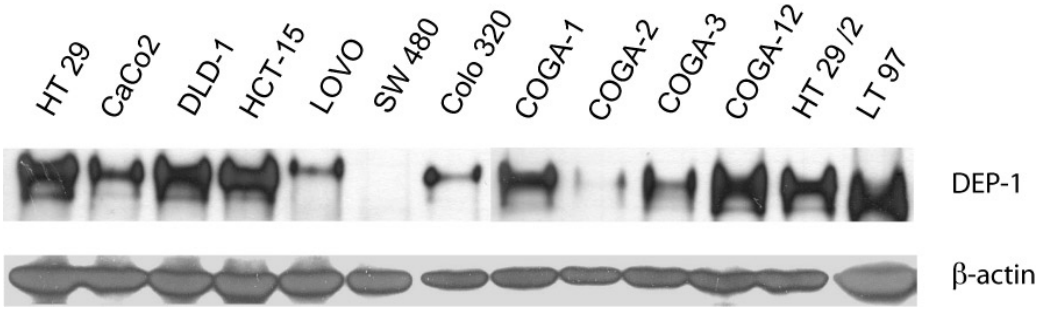


Fig. 3.1a

Fig. 3.1a Expression of DEP-1 in different colon adenoma or carcinoma cell lines.

All cell lines were grown to confluence, and lysates each from a 75 cm^2 flask were subjected to DEP-1 immunoprecipitation and immunoblotting. Lysate aliquots were subjected to immunoblotting for β-actin to compare inputs.

We then performed RT-PCR to know whether the cell line with no detectable protein expression, SW 480, also lacked DEP-1 at the level of mRNA. Fig. 3.1b shows the presence of mRNA for DEP-1 in this cell line SW 480. Nevertheless, this particular cell line SW 480 (as it lacks DEP-1 protein) was chosen for further studies. Re-expression of DEP-1 in these cells should reveal the functional consequences of DEP-1 expression.

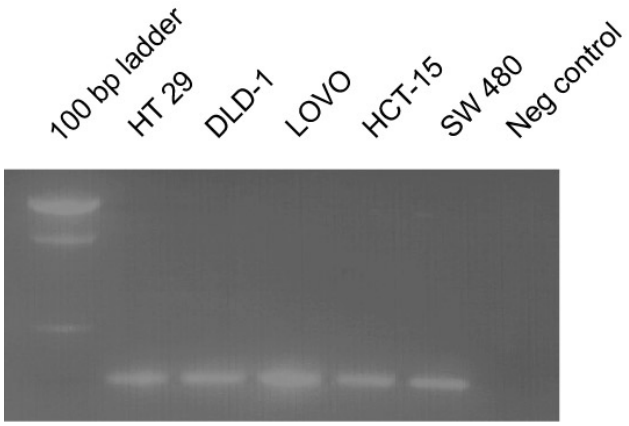


Fig. 3.1b

Fig. 3.1b Detection of mRNA in the colon cancer cell lines.

2 x 10^6 cells were seeded in 6 well plates and lysed. Total RNA was extracted and then cDNA was synthesized. RT-PCR was then performed to detect the presence of DEP-1 mRNA.

3.1.2. Density Dependent Upregulation

DEP-1 has been named "Density-Enhanced Phosphatase" based on its upregulation in dense cultures of human embryonic lung fibroblasts (WI-38) and foreskin fibroblasts (AG1518) (Ostman, Yang et al. 1994). It was unknown whether the density-dependent upregulation also applied to colon epithelial cells.

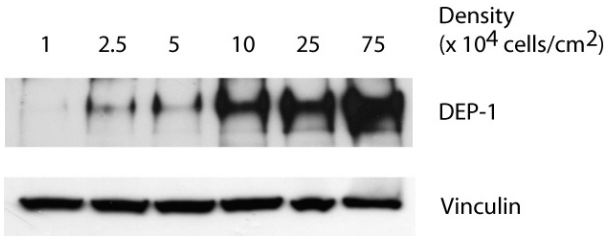


Fig. 3.2

Fig. 3.2 Density dependent upregulation of DEP-1 protein in HT 29.

Cells were seeded at different densities (see Methods) and were lysed the following day. Immunoprecipitations were performed to detect the presence of DEP-1. Loading controls were performed by immunoblotting an equivalent amount of lysates against vinculin.

The density dependent upregulation of DEP-1 was therefore tested in the cell line HT 29. Cells were seeded at different densities (as described in Methods) and lysed the following day.From Fig. 3.2, it is clear that the level of this protein increases upon increase in cell density or as cells approach confluence

3.1.3. Half-life Determination

Half-life is one of the characteristic properties and determines the stability of any protein. Since there was a difference in DEP-1 levels between the sparse and dense cells, we speculated whether changes in half-life might contribute to the observed differences in protein levels. Also it is advantageous to know the half-life of the protein, when working with short interfering RNA (siRNA) molecules. The siRNA molecules target only mRNA, whereas the protein already present should be degraded in order to see any downregulation at the protein level. Therefore we were interested in determining the half-life of DEP-1 and the pulse-chase labelling technique was used for this purpose. Cells were radioactively pulsed with a ^{35}S-labelled mixture of cysteine and methionine for 3 h followed by chasing for different time points. Initially, this approach was attempted with endogenous DEP-1 in HT 29 cells. Due to relatively low labelling of DEP-1 and high background, this system was not successful. Therefore, transiently transfected HEK 293 cells were used instead. From Fig. 3.3, it is clear that the half-life of DEP-1 in these cells is around 12 - 13 hours.

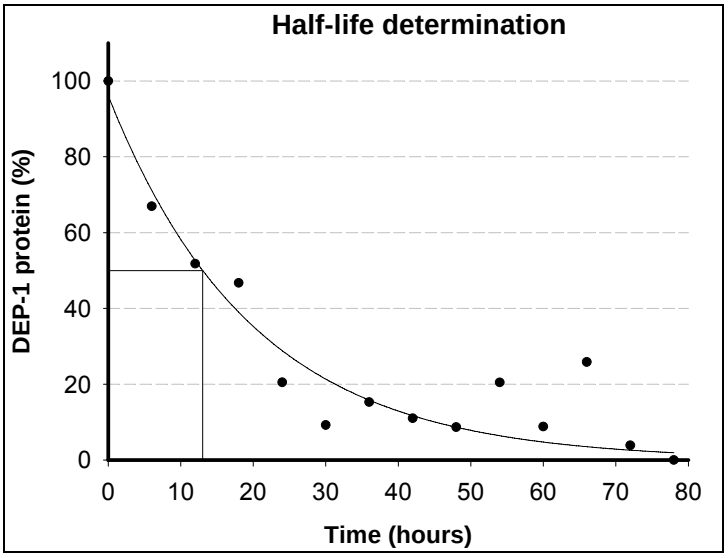


Fig. 3.3

Fig. 3.3 Half-life determination of DEP-1.

HEK 293 cells were transiently transfected with a cDNA encoding the DEP-1 WT. The cells were starved in Cys/Met free medium for one hour after which they were pulsed with a medium containing ^{35}S labeled Cys/Met for three h. Thereafter, the radioactive medium was replaced with non-radioactive medium with all the supplements ("chase"). Cells were then lysed at the indicated time points. Lysates were subjected to IP and separated in a SDS-PAGE. The levels of remaining radioactive DEP-1 at each time point were detected by fluorography. The total DEP-1 protein present at the beginning of the chasing time point (zero hour) was considered as 100% and the graph was plotted accordingly.

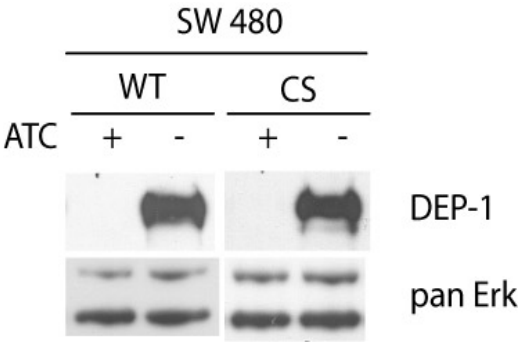
This shows that DEP-1 is a relatively stable protein as the half-life is considerably long. This experiment was performed with transiently transfected cells under subconfluent conditions. However, from this experiment we cannot draw any conclusion about its half-life in sparse and dense cells.

3.2. Characterisation of DEP-1 WT/CS Re-Expressing Cell Lines

3.2.1. Cloning of DEP-1 WT / C1239S in pNRTIS 21 and Generation of Stable Cell Lines

As the initial screening revealed a cell line (SW 480) negative for DEP-1 protein expression, we then aimed to re-express either DEP-1 WT or the catalytically inactive C1239S variant in these cells and then to analyse the resulting phenotypes. Previous work in fibroblast cell lines in our laboratory employed DEP-1 constructs in the tetracycline-regulatable vector pNRTIS 33. For the experiments described here, we subcloned the cDNA for DEP-1 WT or CS from the vector pNRTIS 33 into pNRTIS 21. Both these vectors possess a tetracycline regulated promoter which allows turning off the gene of interest by just adding anhydrotetracycline (Atc) to the growth media. The vector pNRTIS 33 is tricistronic and contains the genes for the tetracycline-repressed transactivator (tTA), the gene of interest (DEP-1) and the neomycin resistance gene, all under the tTA-responsive promoter. Thus, positive cell clones can only be selected in the induced or expressed state. This may lead to failure of the selection of cell clones with phenotypes "adapted" to DEP-1 expression. pNRTIS 21 is bicistronic for tTA and the

gene of interest (i.e., DEP-1), whereas the neomycin expression cassette is under a separate SV-40 promoter. This allows the screening of clones in the absence of any target gene expression and hence was preferred. The sub-cloning was carried out successfully (see Methods), and the resulting constructs (shown in Appendix) were stably transfected into SW 480. Fig. 3.4a shows the generation of stable, inducible DEP-1 WT or CS re-expression. As expected, the target gene is completely repressed in the presence of Atc and expressed without any Atc. Fig. 3.4b shows the phosphatase assay of the lysates of resulting clones. Significant activity was detected only when DEP-1 WT is expressed.



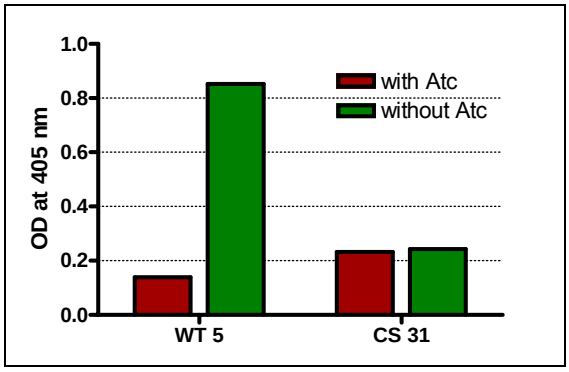


Fig. 3.4a *Fig. 3.4b*

Fig. 3.4 Expression of DEP-1 in SW 480 cells stably transfected with DEP-1 WT and CS variants.

Fig. 3.4a Stably transfected clones (both WT and CS) were seeded with and without Atc (150 ng/ml) and grown for four days. The cells were then lysed (in the absence of sodium orthovanadate) and the lysates were immunoblotted against DEP-1 to detect its expression levels. Detection of total Erk½ was used as a loading control.

Fig. 3.4b 5% of the same lysates were used for the pNpp assay to detect the phosphatase activity of both these WT and CS clones.

Thus, both the wild type and catalytically inactive forms of DEP-1 can be stably and inducibly re-expressed in the cell line SW 480. Moreover, considerable phosphatase activity was detected in lysates only when the wild type is expressed.

3.2.2. Cell Sorting

In general, DEP-1 expression was induced in these clones by culturing the cells without Atc for four days. However, some cells (particularly in later passages) failed to induce DEP-1 expression upon removal of Atc, as was evident from immunostaining (Fig. 3.5). Hence, we decided to enrich those cells that do re-express DEP-1 after induction. For this

purpose, we employed a simple technique called MACS (Magnetic Assisted Cell Sorting). When indicated, the assays described below were performed after MACS enrichment for DEP-1 expressing cells. If no purification is indicated, the assays were performed without prior enrichment but earlier passages were utilized.

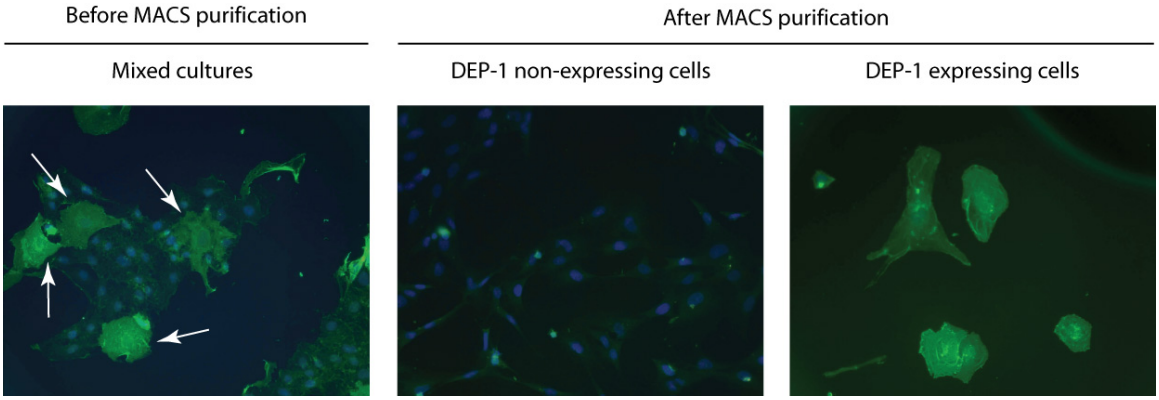


Fig. 3.5

Fig. 3.5 Separation of DEP-1 expressing from the non-expressing cells.

SW 480 cells expressing the DEP-1 WT were cultured in the absence of Atc for four days for complete induction. Cells were then trypsinized and sorted using MACS (see Methods for details). Immunostaining was performed with FITC labelled anti DEP-1 antibody (green channel). Nuclei were stained with DAPI (blue channel). Both the cells before purification (left panel) and after purification (middle and right panel) are shown. DEP-1 expressing cells in the mixed cultures (left panel) are indicated by white arrows. After MACS purification homogenous cultures of DEP-1 expressing (right panel) or non-expressing (middle panel) were obtained.

Thus, pure and homogenous cultures of either DEP-1 expressing and non-expressing cells can be obtained after sorting with MACS.

3.2.3. Proliferation

For thyroid, pancreas and glioma cell lines, DEP-1 re- or over-expression has been shown to lead to growth inhibition. For fibroblasts, controversial results have been obtained. NIH 3T3 clones did not exhibit reduced growth upon induction of DEP-1 expression using the pNRTIS 33 system (Jandt, Denner et al. 2003). In order to know whether the re-expression of DEP-1 confers some growth disadvantage to the colon carcinoma cells, proliferation assays were performed. With the derived SW 480 clones, cell proliferation in presence of wild-type DEP-1 was clearly reduced as compared to cells with suppressed DEP-1 expression (Fig. 3.6a). Inducible expression of the catalytically inactive DEP-1

CS variant had however, no effect on proliferation (Fig. 3.6b), establishing that DEP-1 PTP activity is required for the observed anti-proliferative effect. In the case of DEP-1 WT, proliferation data with prior enrichment of DEP-1 expressing cells is also shown revealing an even more dramatic growth inhibition (Fig. 3.6c).

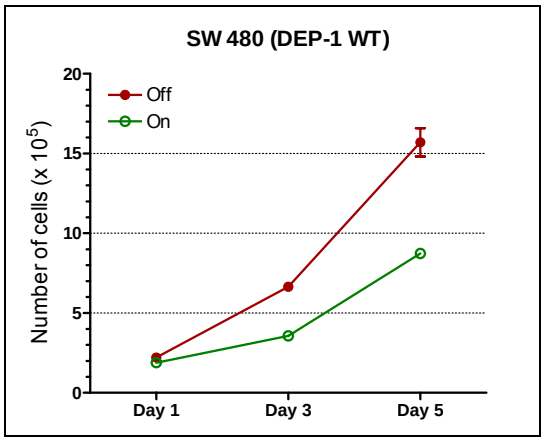


Fig. 3.6a

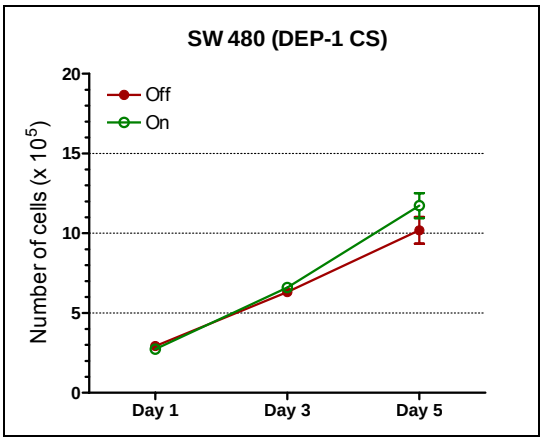


Fig. 3.6b

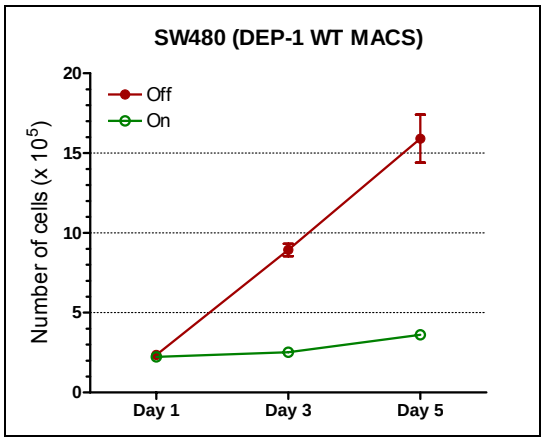


Fig. 3.6c

Fig. 3.6 DEP-1 WT inhibits proliferation of colon cancer cells.

Fig. 3.6a SW 480 clones of DEP-1 WT were seeded in a 6 well plate (100,000 cells/2.0ml/well). They were grown with (designated "off") and without Atc (designated "on") for the respective days after which they were trypsinized and counted using a cell counter. Assays were always performed in triplicates (mean values ± s.d., are shown).

Fig. 3.6b Assays were carried out as described above except that clones of SW 480 expressing DEP-1 CS variants were used.

Fig. 3.6c Proliferation assay (as described above) of the same DEP-1 WT variant, but only DEP-1 expressing cells after sorting with MACS was analysed.

Taken together, DEP-1 is anti-proliferative for the analysed colon carcinoma cells and this effect depends on its catalytic activity.

3.2.4. Migration

DEP-1 is a transmembrane phosphatase with eight fibronectin repeats at the extracellular side. Such motifs suggest its possible participation in cell-matrix adhesion / migration and related signaling mechanisms. Probably in relation to this, effects of DEP-1 on the migration and adhesion of cells have been shown in other cell types. In order to explore similar functions of DEP-1 in the colon epithelial cells, we subjected the DEP-1 re-expressing SW 480 cells to two different types of cell migration assays.

3.2.4.1. Wound healing assays

Wounding assays were performed in the presence of fetal bovine serum (FBS). The re-expression of DEP-1 wild-type led to a clear reduction in cell migration (Fig. 3.7). Fig. 3.7a shows examples for the microscopic observation of the cell migration in SW 480 cells expressing DEP-1 WT. Pictures were made immediately after wounding (0 h) and also at the completion of the assay (after 24 h). These assays clearly revealed that the expression of DEP-1 strongly inhibits cellular migration. Five random fields were also quantified and the results are shown in Fig. 3.7b. Inhibition of cell migration was highly significant. In contrast, the DEP-1 CS mutant had no effect on cell migration (Fig. 3.7c). Inhibition of cell migration, thus, also depends on DEP-1 PTP activity.

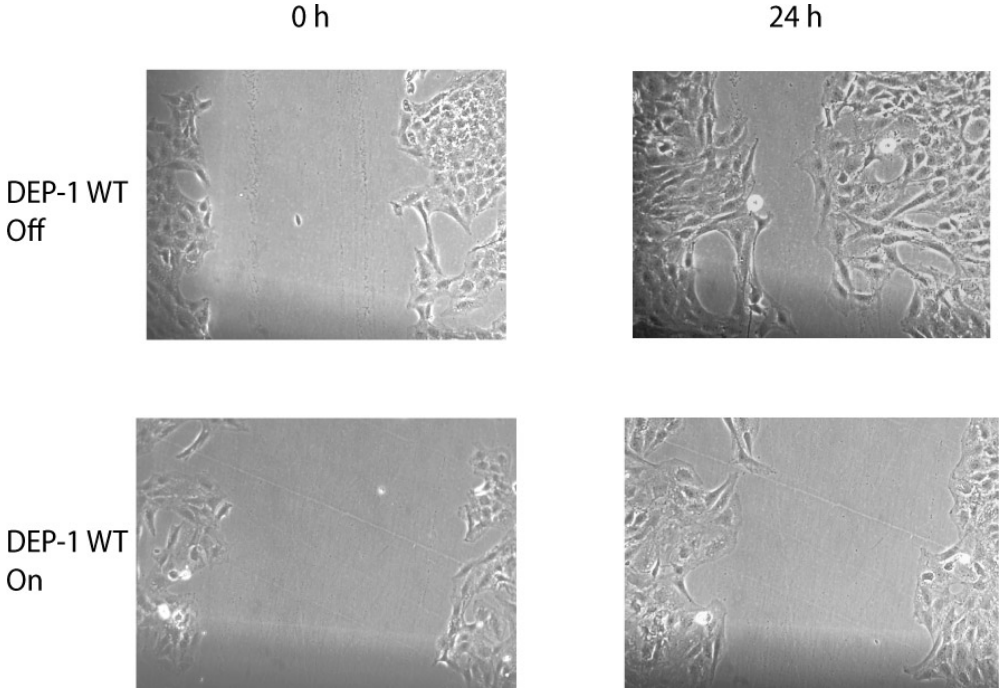


Fig. 3.7a

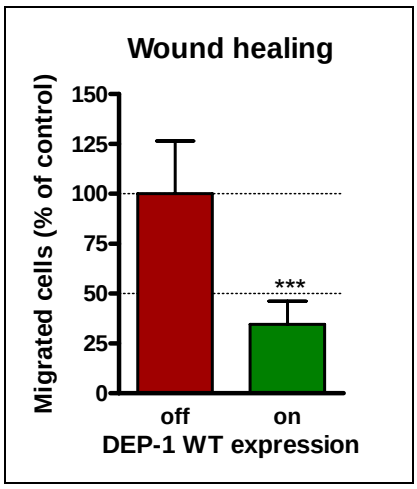


Fig. 3.7b

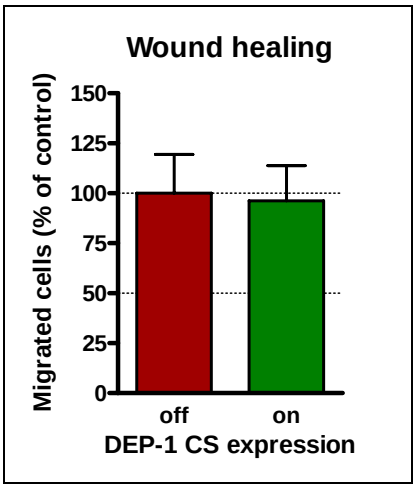


Fig. 3.7c

Fig. 3.7 DEP-1 WT inhibits migration of colon cancer cells in wound healing assays.

Fig. 3.7a Early passages of DEP-1 wild-type-expressing cells in the induced (on) or repressed (off) state were seeded in 6 well plates, allowed to grow to confluence and starved overnight. The cell layer was wounded by scratching with a sterile 1.0 ml pipette tip. Medium containing 10% fetal bovine serum was added and the plates were incubated for further 24 h.

*Fig. 3.7b Quantification of five different positions in three wounding experiments (mean values± s.d., ***P<0.001 by t-test).*

Fig. 3.7c Same experiment as in 3.7a and 3.7b, but using DEP-1 CS-expressing cells. Only the quantification is shown.

Thus, the expression of DEP-1 WT in SW 480 clearly inhibited migration in wound healing assays.

3.2.4.2. Transwell assays

Since wounding assays were performed over a period of 24 h, cell proliferation may contribute to repopulation of the wound. To exclude the effects of proliferation, we therefore also conducted a different type of migration assay with the modified Boyden chamber system. These assays were scored after 6 h, excluding a contribution of proliferation to the observed inhibiton of migration. Consistent with the results in the wounding assays, wild-type DEP-1 re-expression significantly reduced migration towards FBS (Fig. 3.8a), whereas DEP-1 CS apparently had no effect on it (Fig. 3.8b).

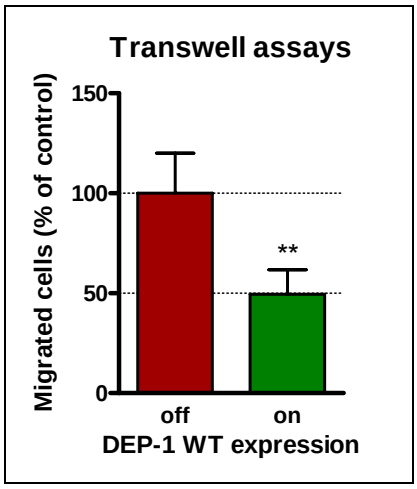


Fig. 3.8a

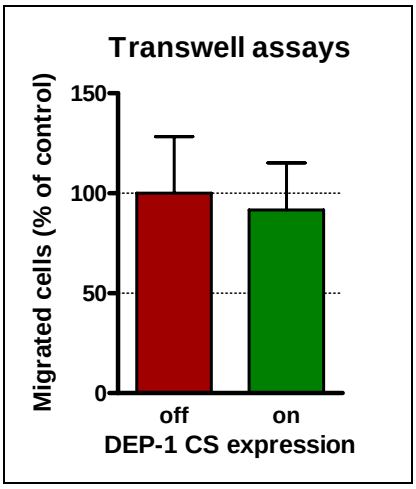


Fig. 3.8b

Fig. 3.8 DEP-1 WT, but not CS inhibits migration of colon cancer cells in transwell assays.

*Fig. 3.8a Early passages of DEP-1 wild-type-expressing cells in the induced (on) or repressed (off) state were seeded on top of a collagen-coated membrane with 8 µM pore-size and cells migrating to the bottom side (media containing 10% FBS) were scored after 6 h by staining with crystal violet and counting in the microscope. Five different fields randomly chosen were counted and quantified (mean values±s.d., **P<0.01 by t-test).*

Fig. 3.8b Same experiment as in Fig. 3.8a except that DEP-1 CS-expressing cells were used.

Since re-expression of DEP-1 inhibits migration significantly in wound healing as well as transwell assays, the anti-migratory role of DEP-1 is firmly established.

3.2.4.3. Effect of various motogens

In order to explore whether the anti-migratory effect of DEP-1 depends on the motogen, it is mandatory to know the responsiveness of this cell line (SW 480) towards a range of motogens. Hence, several common motogens including lysosphosphatidic acid (LPA), sphingosine-1-phosphate (S1P), Stromal Cell Derived Factor - 1 alpha (SDF-1α) and Hepatocyte Growth Factor (HGF) were tested in wounding assays and their migratory potential was scored. As depicted in Fig. 3.9, LPA at a concentration of 10 µM stimulated migration nearly as efficient as FBS whereas the other potential motogens did not reveal any migratory response.

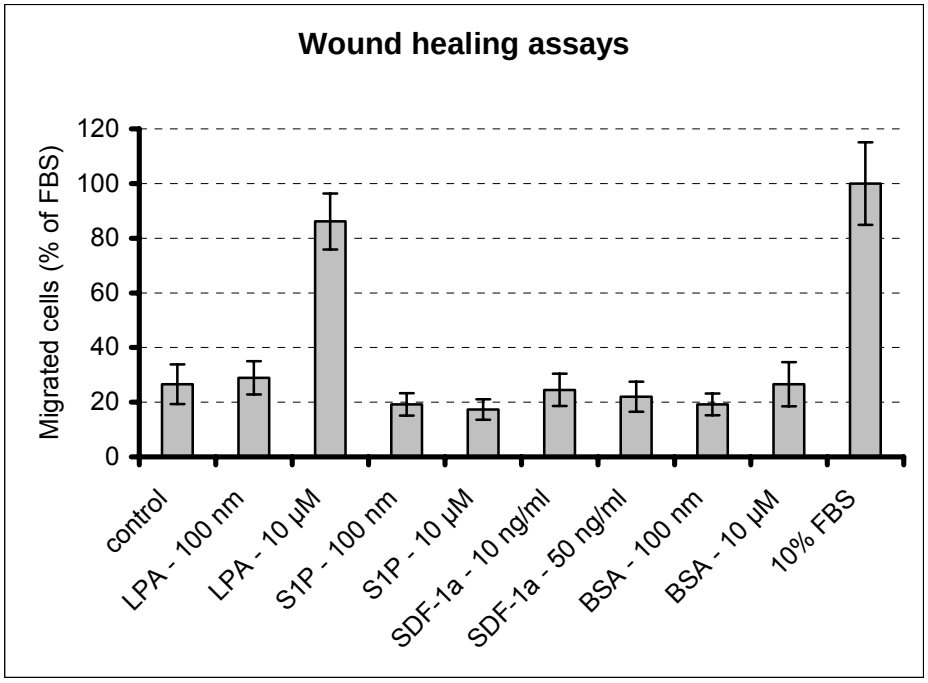


Fig. 3.9

Fig. 3.9 Effects of various motogens on the migration of SW 480 cells.

The parental cells SW 480 were seeded in a 6 well plate and allowed to grow to confluence. Wounding assays were performed as described in Fig 3.7a. Following scratching, the cells were stimulated with various motogens at the indicated concentrations. 24 h later their migratory response was scored.

Thus, LPA can also be employed as a motogen in these SW 480 cell lines which will further shed more light on the anti-migratory role of DEP-1.

LPA as a motogen for cellular migration

Since LPA at a concentration of 10 µM stimulated cell migration in the SW 480 cell line, transwell migration assays were performed to determine the effect of DEP-1 WT or CS expression on LPA stimulated migration. When stimulated with 10 µM LPA, the re-expression of DEP-1 WT profoundly inhibited migration (Fig. 3.10a) whereas the CS mutant had no effect (Fig. 3.10b).

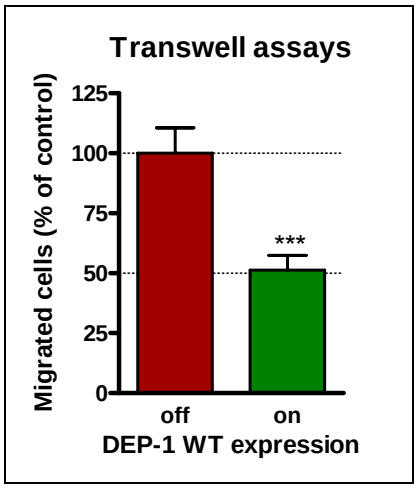


Fig. 3.10a

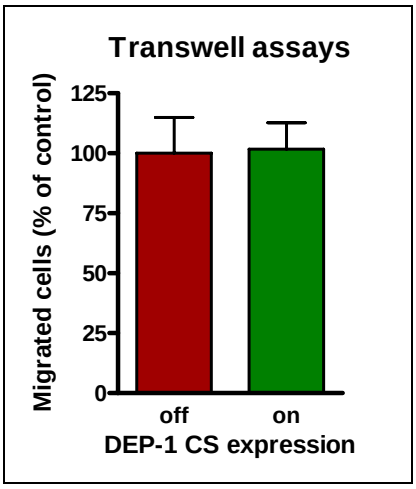


Fig. 3.10b

Fig. 3.10 DEP-1 WT inhibits also migration stimulated by LPA.

*Fig. 3.10a Transwell assays were carried out as described in Fig. 3.8a except that 10 µM LPA in RPMI was used as motogen (mean values±s.d., ***P<0.001).*

Fig. 3.10b Similar assays as in Fig. 3.10a except that DEP-1 CS expressing clones were used.

HGF as a motogen for cellular migration

Hepatocyte Growth Factor (HGF), popularly referred to as "scatter factor", is an established motogen in several cellular systems. Even though HGF did induce migration in these cell lines under investigation, the extent of migration induced was less than that induced by FBS or LPA (not shown). When HGF (50 ng/ml) was used as a motogen in wound healing assays, the re-expression of DEP-1 again significantly inhibited the migration (Fig. 3.11).

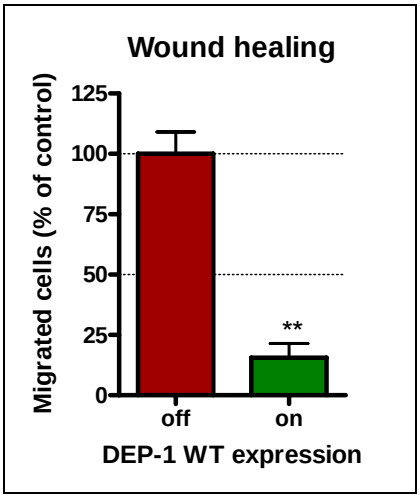


Fig. 3.11

Fig. 3.11 DEP-1 WT inhibits migration stimulated by HGF.

*Early passages of SW480 cells expressing the DEP-1 WT in the induced (on) or repressed (off) state were seeded in 6 well plate and wound healing assays were performed as described earlier in Fig. 3.7a. 50 ng/ml of HGF was used as motogen and 24 h later, five randomly chosen fields were counted and quantified (mean values ±s.d., **P<0.01).*

In conclusion, DEP-1 inhibits the migration of cells, not only stimulated by FBS but also the one stimulated by LPA and HGF. This effect depends on its catalytical activity.

3.2.5. Adhesion

The effect of DEP-1 in cell migration could possibly be linked to altered adhesion of the cells to the extracellular matrix. For NIH 3T3 fibroblasts and PAE cells, enhanced adhesion as a consequence of DEP-1 expression has been shown (Jandt, Denner et al. 2003). In order to test this hypothesis, adhesion assays were carried out in two different ways. With the first method, we checked whether the cells re-expressing DEP-1 can attach faster than their non-expressing counterparts. As shown in Fig. 3.12a, there was no difference in the adhesion rate of the cells towards the extracellular matrix. With the second method, we scored the detachment rate of cells from the substratum either by simply washing off the cells with Ca^{2+} and Mg^{2+} free PBS alone, or in combination with a mild proteolytic treatment using Accutase. Consistent with the first method, again there was no difference in the detachment rate of the cells either by PBS alone (not shown) or in combination with Accutase (Fig. 3.12b).

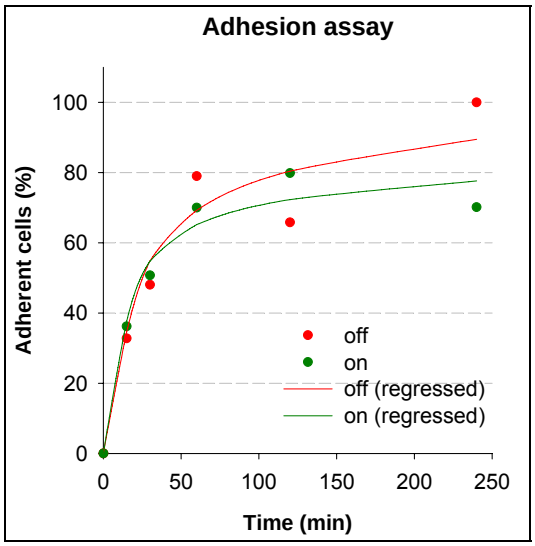


Fig. 3.12a

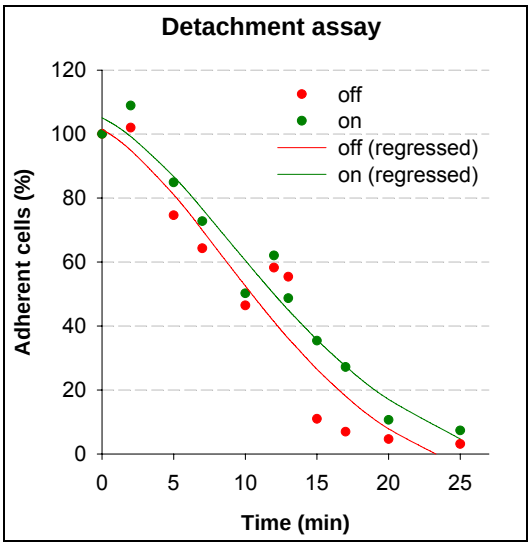


Fig. 3.12b

Fig. 3.12 DEP-1 WT does not play any role in cell-matrix adhesion.

Fig. 3.12a Early passages of SW 480 cells expressing DEP-1 WT were grown either in the presence or absence of Atc for four days. They were trypsinized and kept in suspension for 30 min. The cells were then seeded in collagen coated 96 well plates. At the indicated time points, non-adhered cells were removed and the adhered cells were quantified by staining with crystal violet as described in Methods. The maximum attachment of DEP-1 WT non-expressing cells reached after 4 h was considered as 100%.

Fig. 3.12b SW 480 cells expressing DEP-1 WT were grown without Atc for four days and were purified using MACS. The resulting DEP-1 expressing and non-expressing cells were seeded in 24 well plates (without and with Atc respectively) and allowed to grow for additional two days. After that, the cells were washed once with PBS followed by detaching them with Accutase. At the indicated time points, detached cells were washed away and the remaining adherent cells were scored as describe above. Cells that were attached to the substratum before the beginning of the assay (at time - 0 min) were considered as 100%.

Thus, the re-expression of DEP-1 does not play any role in adhesion at least in the cell lines investigated.

3.2.6. Immunostaining

The cells re-expressing DEP-1 WT, when viewed under the microscope were larger and exhibited a more flattened morphology than their non-expressing counterparts. In addition, DEP-1 mediated cytoskeletal changes were proposed in the case of fibroblasts (Kellie, Craggs et al. 2004). To evaluate possible cytoskeletal rearrangements in the DEP-1 expressing SW 480 colon epithelial cells, we stained the cells with fluorescently labelled phalloidin which stains the filamentous actin.

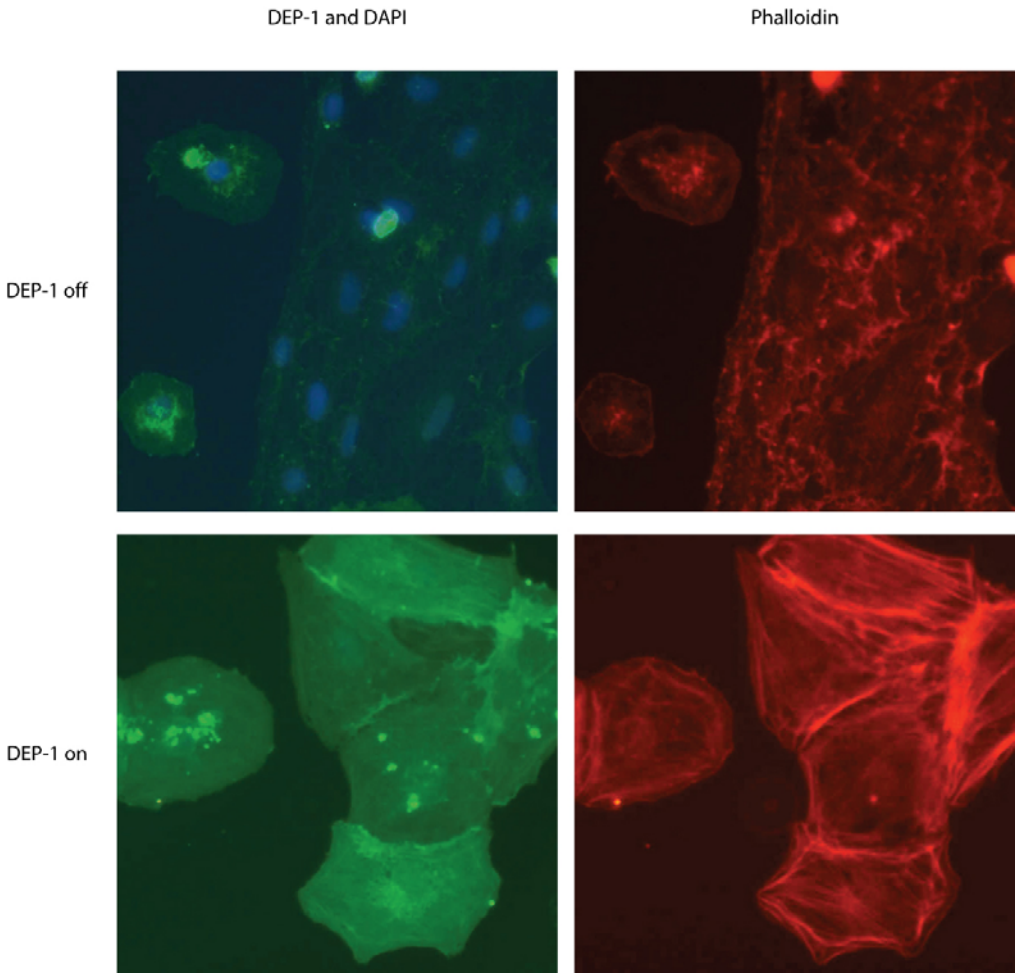


Fig. 3.13

Fig. 3.13 Effect of DEP-1 expression on actin cytoskeleton.

Phalloidin staining of SW 480 cells either expressing (on) or non-expressing (off) wild type DEP-1. Cells were seeded on collagen coated coverslips and staining was carried out as described in the Methods. Cells were stained with anti DEP-1 antibodies followed by DTAF-labelled anti mouse-IgG (green channel), TRITC-labelled phalloidin for staining of actin (red channel), and DAPI to visualize nuclei (blue channel). Upper panel: Cells that do not express DEP-1 (off). Lower panel: Cells expressing wild-type DEP-1 (on).

From Fig. 3.13, it is clear that the DEP-1 re-expressing cells (on) exhibit a different distribution of filamentous actin, typically characterized by cortical actin bundles in proximity to the cell periphery. On the other hand, there was no such organized actin bundles when DEP-1 is not expressed (off). Fig. 3.14 shows a magnified image clearly illustrating the bundling of filamentous actin due to re-expression of DEP-1 in SW 480 cells.

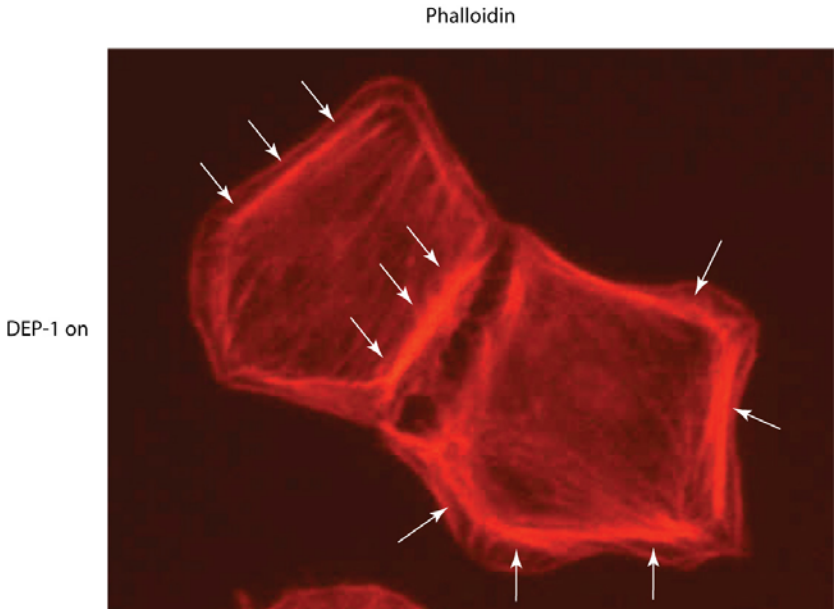


Fig. 3.14

Fig. 3.14 A magnified view of SW 480 cells expressing DEP-1 WT and stained with phalloidin.

Same cells as described in Fig. 3.13, but a magnified image of only DEP-1 WT expressing cells stained with phalloidin is shown. This clearly reveals the presence of peripheral actin bundles (white arrows).

Given the migratory phenotype and the striking cytoskeletal changes, we expected also a possible differential distribution of the molecules involved in focal adhesion or focal complexes. There are several molecules in these focal complexes that are critical for migration. Molecules such as vinculin, talin, paxillin, p120 catenin, α-actinin as well as focal adhesion kinase (FAK) are either directly or indirectly connected to the actin cytoskeleton. In brief, following integrin engagement to the extracellular matrix, c-Src is activated and simultaneously focal adhesion kinase (FAK) is also recruited. This results in the phosphorylation of paxillin which can then activate the small GTPase Rac. The interplay between these above mentioned molecules is important in deciding the migratory phenotype of the cell. Hence immunostaining was performed for a few of them such as vinculin and paxillin. Since DEP-1 expressing cells exhibited hyperphosphorylation of paxillin (as revealed in immunoblotting - see below), we have also stained the cells for phospho-paxillin. As shown in Fig. 3.15, in the case of DEP-1 WT re-expressing cells, the molecules involved in focal adhesion were strongly

concentrated only at certain regions of the membrane. But in their non-expressing counterparts, at least vinculin and paxillin were distributed throughout the membrane.

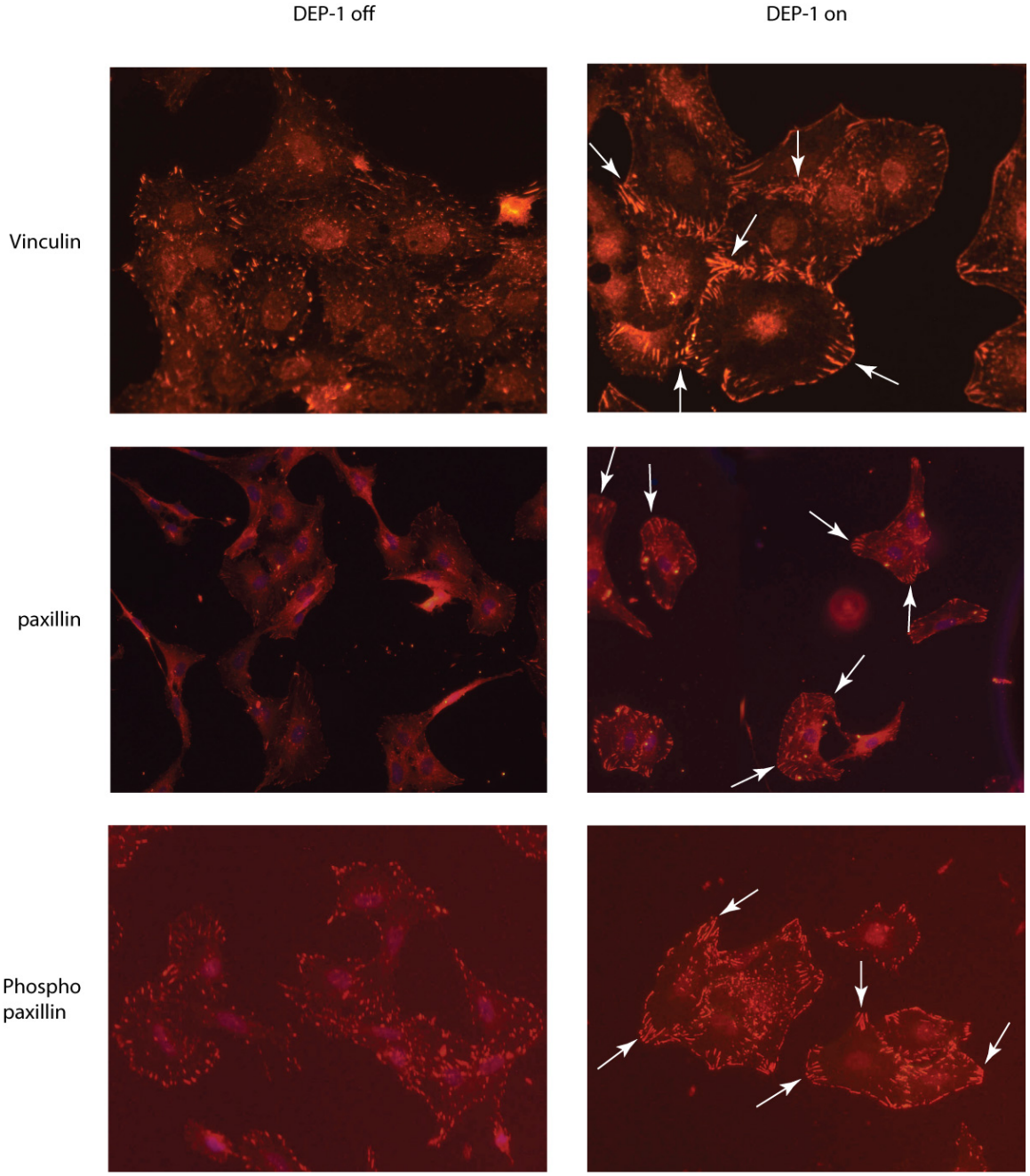


Fig. 3.15

Fig. 3.15 Effect of DEP-1 expression on distribution of vinculin, paxillin and phospho paxillin.

SW 480 cells expressing DEP-1 WT were purified through MACS and seeded on collagen coated coverslips. Staining was carried out with antibodies directed against vinculin (top panel), paxillin (middle panel) or phospho paxillin (lower panel). This was followed by

the addition of either rabbit or mouse secondary antibody (cy 3 labelled) which was visualized using the red channel. Nuclei were stained with DAPI (blue channel). Left panel: Cells that do not express DEP-1 (off). Right panel: Cells expressing wild-type DEP-1 (on). Clustering of the molecules is indicated with white arrows.

Taken together, the re-expression of DEP-1 led to strong re-arrangements of actin cytoskeleton, associated with clustering and presumably activation of focal adhesion complexes, indicated by presence of phospho-paxillin.

3.2.7. Apoptosis Assay

DEP-1 has been proposed as a tumor suppressor protein. One known function of some tumor suppressors is to sensitize the cells towards apoptosis. In a very recent siRNA screen, the effects of depletion of kinase and phosphatase expression on apoptosis induced by cytotoxic drugs such as cisplatin, taxol and etoposide were analysed. In this screen using HeLa cells, DEP-1 was scored as an apoptosis-promoting protein (MacKeigan, Murphy et al. 2005). We therefore wondered whether re-expression of DEP-1 in colon epithelial cells can also have a role in apoptosis regulation. Apoptosis was induced using staurosporine, and ELISA based assays were performed. From Fig. 3.16a, it is clear that the re-expression of DEP-1 WT rendered the cells more apoptotic whereas the catalytically inactive DEP-1 mutant had no effect (Fig. 3.16b).

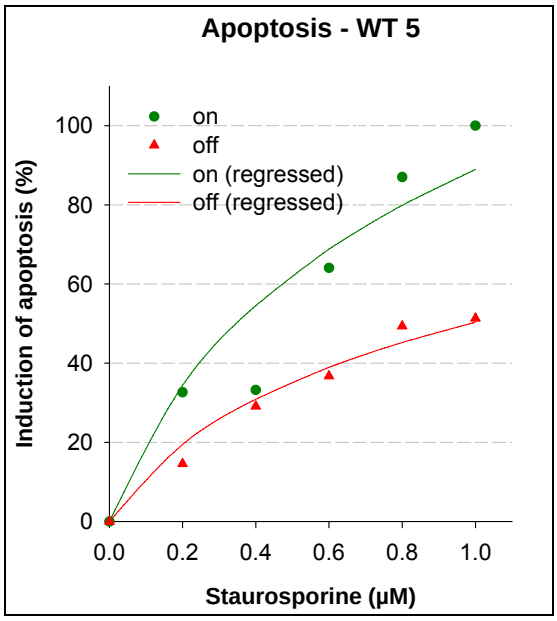


Fig. 3.16a

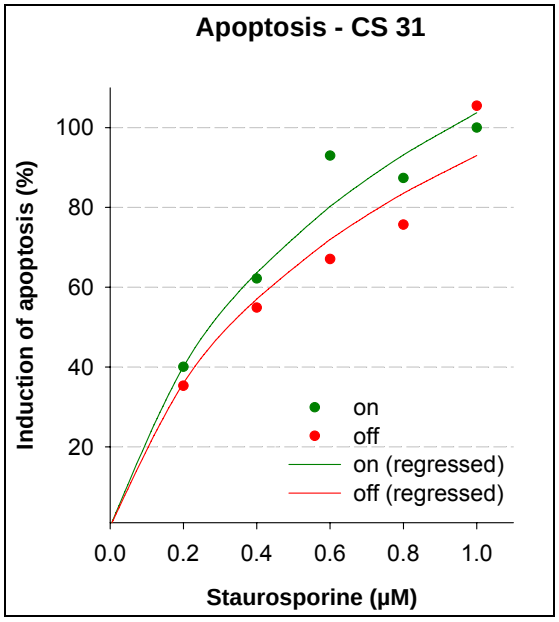


Fig. 3.16b

Fig. 3.16 DEP-1 WT sensitizes the cells towards apoptosis.

Fig. 3.16a DEP-1 WT expressing SW 480 cells were cultured in the absence of Atc for four days and purified with MACS. DEP-1 non-expressing and expressing cells were then seeded in 24 well plates and treated with the indicated concentrations of staurosporine for four h. They were then lysed and lysates corresponding to 1 x 10^3 cells were used for and apoptosis detecting ELISA (see Methods). The color development was measured at 405 nm. Apoptosis induction by 1 µM Staurosporine in the DEP-1 expressing cells was considered as 100%.

Fig. 3.16b SW 480 cells expressing DEP-1 CS were used and similar assays were carried out as described in Fig. 3.16a.

These experiments show that the sensitization of the cells towards apoptosis also depends on its catalytical activity as only in wild type expressing cells apoptosis is promoted. It seems therefore likely, that one or more DEP-1 targets in the SW 480 cells have an anti-apoptotic function.

3.3. Signaling Mechanisms Evoked by Re-Expression of DEP-1 in SW 480 Cells

3.3.1. Phosphorylation Status of Paxillin, FAK and PLCγ – Molecules Involved in Migration

Since there was a strong inhibition of migration which is also associated with changes in the distribution of focal adhesion molecules, the phosphorylation status of proteins involved in focal adhesion proteins was analysed. As shown in Fig. 3.17a, the cells re-expressing DEP-1 WT exhibited a strong hyperphosphorylation of paxillin at Tyr^{118}. In sharp contrast, DEP-1 re-expression led to nearly complete dephosphorylation of PLCγ at Tyr^{783}. In the case of Focal Adhesion Kinase (FAK), site specific hyperphosphorylation was detected at Tyr^{408}, Tyr^{861} and Tyr^{925} whereas other sites (Tyr^{566} and Tyr^{567}) failed to reveal any significant changes (Fig. 3.17b). Analysis of the phosphorylation status of c-Src kinase revealed a small increase of phospho-tyrosine in the activation loop (Tyr^{418}) whereas no significant dephosphorylation at the inhibitory site (Tyr^{529}) was evident (Fig. 3.17c). It should be noted that in all the cases, even though the cells were stimulated with FBS, all described changes evoked by DEP-1 re-expression were also evident in unstimulated cells. Moreover, none of these changes were revealed in cells expressing the

catalytically inactive DEP-1 CS variant, indicating the importance of its phosphatase activity.

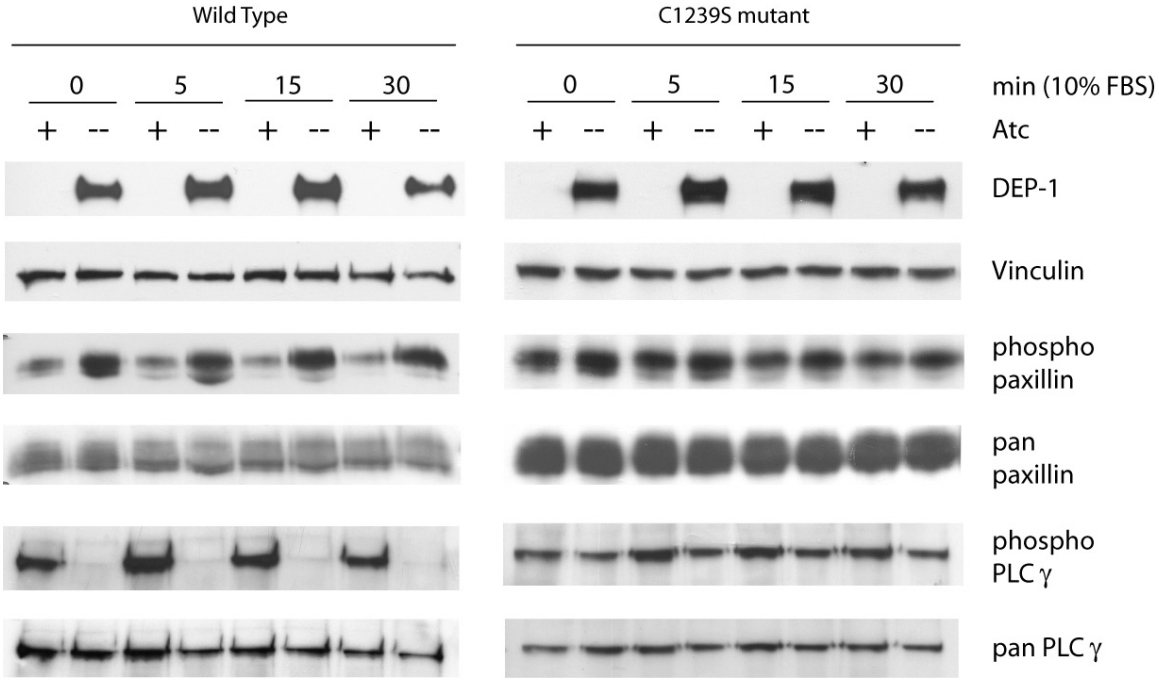


Fig. 3.17a

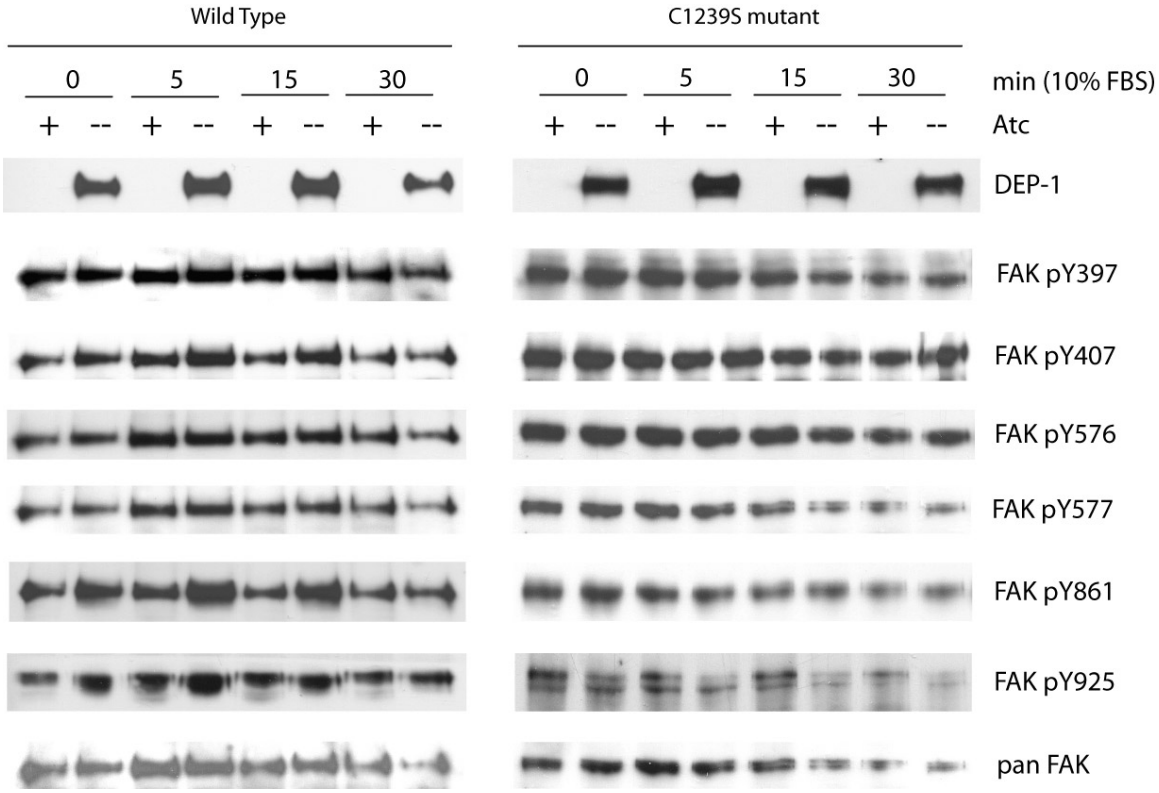


Fig. 3.17b

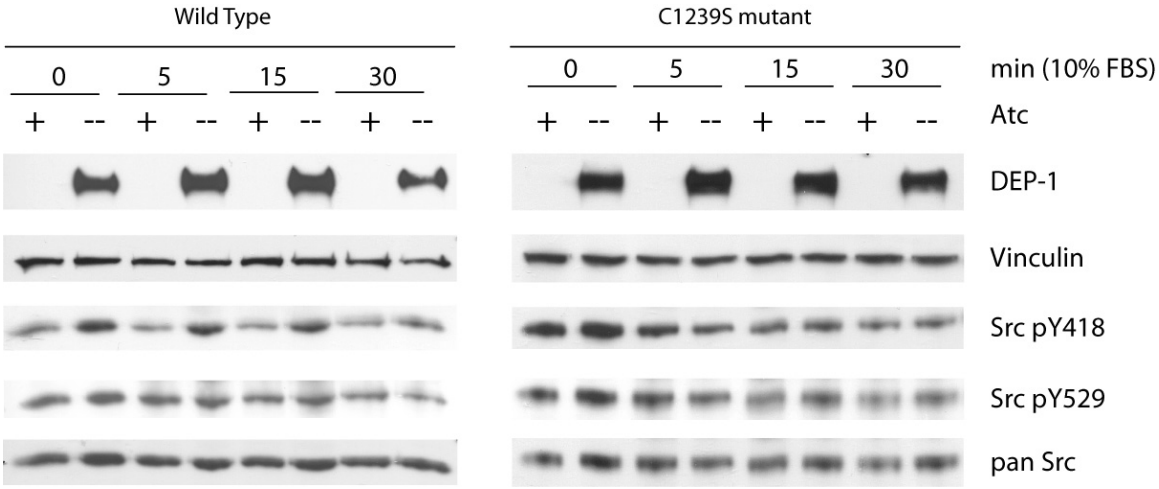


Fig. 3.17c

Fig. 3.17 Expression of DEP-1 WT causes hyperphosphorylation of paxillin, of some phospho-tyrosyl sites in FAK and a complete dephosphorylation of PLCγ.

Fig. 3.17a DEP-1 WT or CS expressing SW 480 cells were cultured either in the presence or absence of Atc (as indicated) and DEP-1 expressing cells were enriched using MACS. 500,000 cells/6.0 cm dishes were seeded and allowed to grow for 2-3 days. Cells were starved overnight, followed by stimulation with 10% FBS for the indicated time points after which they were lysed. Lysates were analysed for their DEP-1 expression. Loading controls were performed by immunoblotting against vinculin. Lysates were also probed to detect phospho-paxillin and phosphor-PLCγ with corresponding antibodies. The same membrane was stripped and immunoblotted to detect the total levels of paxillin and PLCγ species.

Fig. 3.17b The same lysates were used to detect site-specific phosphorylation of FAK. Site specific antibodies that recognize different tyrosyl phosphorylated species of FAK were employed. The total levels of FAK were also detected using an antibody that detects FAK irrespective of its phosphorylation status.

Fig 3.17c Same lysates were probed to detect the phosphorylation status of c-Src. Both activating Tyr^{418} and inhibitory Tyr^{529} tyrosine residues were detected using antibodies which specifically recognize the corresponding phosphorylated sites. Total c-Src levels were also detected.

Thus, the re-expression of DEP-1 leads to hyperphosphorylation of paxillin and some tyrosyl sites in FAK, and is associated with complete dephosphorylation of PLC γ. These changes may be due to the elevated Src activity.

3.3.2. Phosphorylation Status of Erk and Akt – Molecules Involved in Proliferation and Survival

Since DEP-1 is anti-proliferative and pro-apoptotic in the SW 480 cells, the activation status of two signaling molecules which are often actively involved in these processes - Erk (proliferation) and Akt (apoptotis) were analysed. As shown in Fig. 3.18, the re-expression of DEP-1 WT caused hypophosphorylation of Akt (independent of stimulation). Even though phosphorylation of Erk is inducible with FBS, there was no significant difference between phospho-Erk levels in the DEP-1 expressing cells and their non-expressing counterparts. These results were also validated with cells expressing the DEP-1 CS mutant, and again no changes were observed.

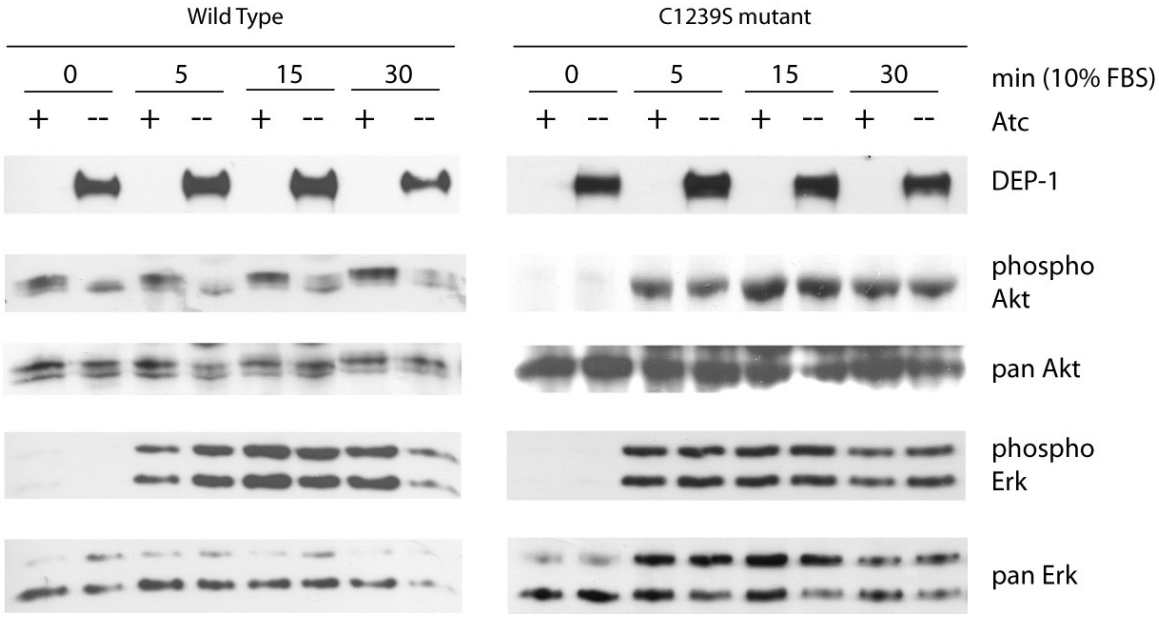


Fig. 3.18

Fig. 3.18 Expression of DEP-1 causes decreased phosphorylation of Akt.

Lysates were prepared as described in Fig. 3.17a. They were probed to detect the phosphorylation status of Akt and Erk. Loading controls were performed by detecting total Erk1/2 and Akt levels after stripping of the same membrane.

Thus, the re-expression of DEP-1 is associated with hypophosphorylation of Akt whereas phospho-Erk levels, induced as a result of serum stimulation were not affected.

3.3.3. Effect of DEP-1 on Signaling Induced by Various Cellular Stimuli

In order to understand the effect of DEP-1 on different pathways, it is essential to know which factors can induce phosphorylation cascades (potential motogens or mitogens) in SW 480 cells. Hence a range of factors (EGF, HGF and LPA) were screened and the phosphorylation status of Erk was initially used to score the cellular signaling response. As is evident from Fig. 3.19, Hepatocyte Growth Factor (HGF) can induce similar levels of Erk phosphorylation as FBS, followed by Lysophosphatidic Acid (LPA), whereas Epidermal Growth Factor (EGF) didn't induce any phosphorylation. Thus, EGF is potentially not a relevant stimulus of SW 480 cells and was not further investigated. In contrast, HGF appears as a potentially important factor in this cell line.

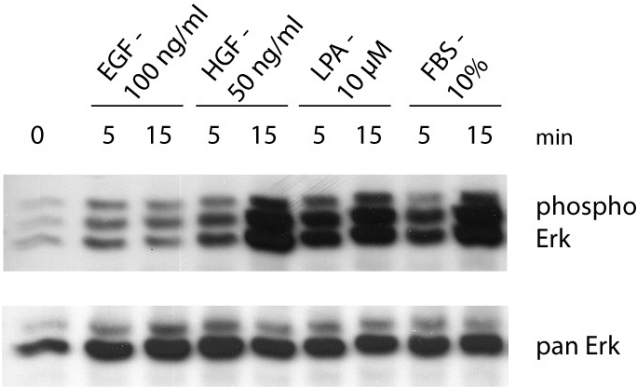


Fig. 3.19

Fig. 3.19 Effect of different factors on Erk1/2 activation in SW 480 cells.

The parental SW 480 cells were seeded in 6 well plates, starved overnight and stimulated with the indicated factors for 5 and 15 min. The cells were then lysed and immunoblotted with phospho-Erk antibody. Loading controls were performed by detecting total Erk levels. Unstimulated cells were shown in the first lane as a control for stimulation.

Starting from these observations, the effects of DEP-1 expression on LPA and HGF-induced signaling were analysed more closely.

3.3.3.1. Lysophosphatidic acid (LPA)

DEP-1 expressing and non-expressing cells were starved overnight and stimulated with 10 µM LPA for the indicated time points. As is evident from Fig. 3.20, LPA induced a similar level of Erk phosphorylation as FBS. Hyper-phosphorylation of paxillin, hypo-phosphorylation of Akt was also observed and again both these effects were independent

of LPA stimulation. Phosphorylation of Erk following LPA stimulation was again not affected due to the re-expression of wild type DEP-1.

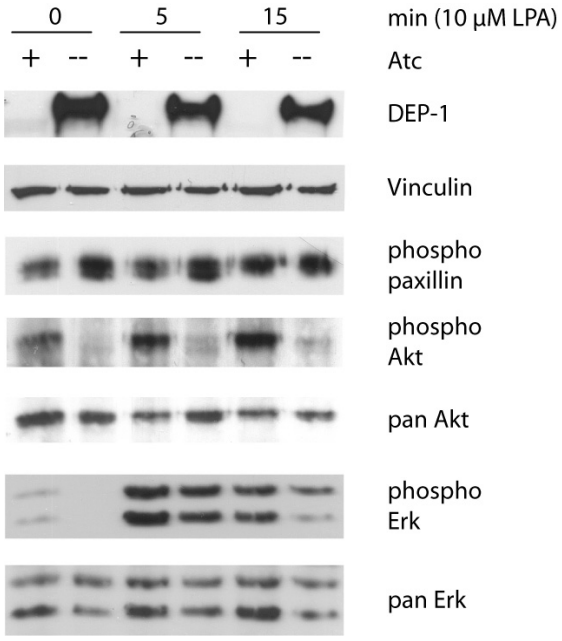


Fig. 3.20

Fig. 3.20 Effect of DEP-1 expression on LPA-induced signaling in SW 480 cells.

The experiment was performed as described in Fig. 3.17a except that 10 µM LPA was used for stimulation. Lysates were probed to detect levels of phosphorylated Akt, Erk and paxillin. Controls were shown by reprobing the membrane with antibodies which detect total levels of Erk / Akt.

Thus, the effect of DEP-1 expression upon stimulation with LPA mirrors, the one upon FBS stimulation in these cells.

3.3.3.2. Hepatocyte Growth Factor (HGF)

HGF stimulation led to phosphorylation of its receptor HGFR (c-Met) which was easily detectable at the level of cell lysates. Expression of DEP-1 WT led to a complete dephosphorylation of the receptor at $Tyr^{1234,\ 1235,\ 1236}$ as detected with the phospho-tyrosine antibodies directed against these residues. Also, receptor levels appeared reduced upon DEP-1 re-expression (Fig. 3.21). Paxillin hyper-phosphorylation and Akt hypo-phosphorylation in the presence of DEP-1 were again observed independent of stimulation (similar to FBS and LPA stimulation – compare Fig. 3.17, Fig. 3.18 and Fig. 3.20). In contrast, the effect on the phosphorylation status of Erk was very different. Phosphorylation of Erk was completely undetectable in the case of DEP-1 WT expressing

cells after HGF stimulation. Consistent with FBS stimulation, the expression of the CS mutant hardly revealed any changes in the phosphorylation status of all these proteins following HGF stimulation emphasizing again the essential role of the phosphatase activity of DEP-1 (not shown).

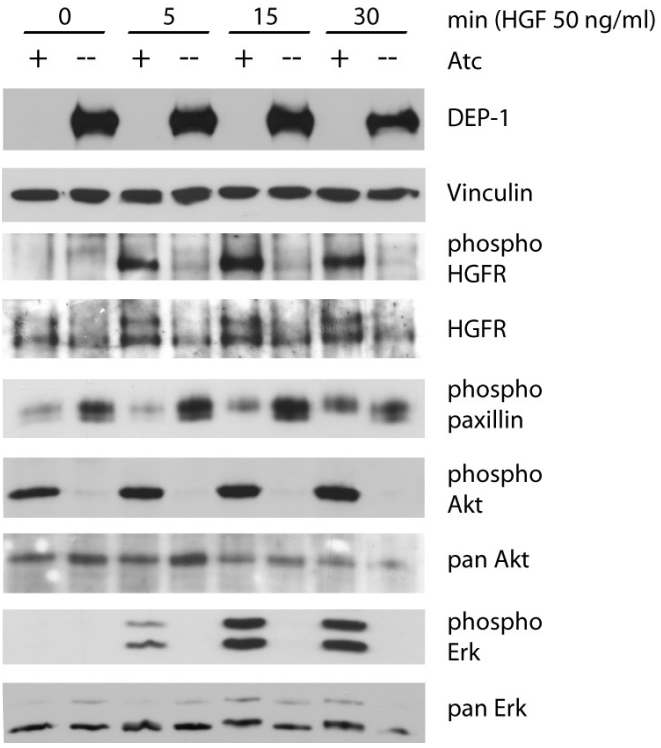


Fig. 3.21

Fig. 3.21 DEP-1 WT expression leads to dephosphorylation of HGFR (c-Met).

The experiment was performed as described in Fig. 17 except that 50 ng/ml of HGF was used for stimulations. The phosphorylation status of HGFR and of some signaling proteins (paxillin, Akt and Erk) were also analysed. Control blots were performed after stripping the same membrane to detect the total levels of paxillin, Akt and Erk.

Thus, re-expression of DEP-1 WT in SW 480 cells upon HGF stimulation can antagonize not only its receptor (HGFR) but also their downstream effectors (Akt, Erk).

3.3.4. Pull-downs of the Small GTPases Rho and Rac

Since DEP-1 expressing cells showed a hypo-migratory phenotype associated with cortical stress fibre formation, we speculated whether this could be mediated via small GTPases such as Rho or Rac. We performed pull downs to see the active fraction of Rho or Rac (GTP bound forms) against the total Rho and Rac. As shown in Fig. 3.22, there was no obvious difference in the activation status of Rac, which was constitutively high,

due to the re-expression of DEP-1 WT. However, there was a small difference in the activation state of Rho when DEP-1 WT was expressed (again independent of stimulation) which could be related to the observed changes in actin cytoskeleton.

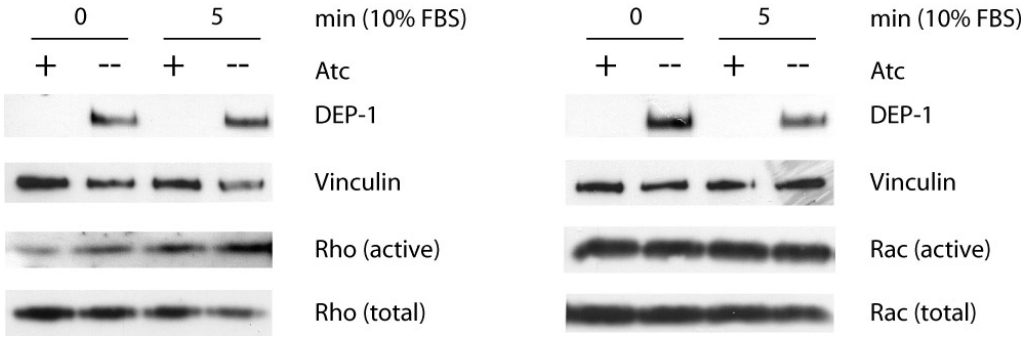


Fig. 3.22

Fig. 3.22 Re-expression of the DEP-1 WT causes activation of the small GTPase Rho but not Rac.

SW 480 cells expressing the DEP-1 WT were purified through MACS and seeded as described in Fig. 3.17a. They were either left unstimulated or stimulated for 5 min with 10% FBS. Cells were then lysed in the presence of corresponding binding domains and GSH beads were used to pull down the active fractions of Rho / Rac as described in Methods. Lysate aliquots were subjected to immunoblotting to detect the total levels of Rho or Rac and to confirm DEP-1 expression.

Thus, the observed changes in migration and actin cytoskeleton of the DEP-1 re-expressing cells may be due to the upregulation of Rho GTPases.

3.4. shRNA Mediated Suppression of DEP-1

The re-expression of DEP-1 WT showed an anti-proliferative, anti-migratory and pro-apoptotic phenotype accompanied by several changes in the signaling molecules. This approach may, however, lead to over-interpretation of some effects, due to supraphysiological levels of DEP-1. Knockdown of endogenous DEP-1 expression is a complementing approach. We therefore wondered, whether some of the phenotypes could be recapitulated by suppressing endogenous DEP-1. This should greatly facilitate to understand the physiological relevance of DEP-1 in colon cells. DEP-1 suppression in a suitable cell line was carried out using a shRNA construct that produces a 64 nucleotide (nt) short hairpin RNA (shRNA) which upon processing targets the endogenous DEP-1 mRNA.

3.4.1. Testing of shRNA Constructs in Transient Transfections in HEK 293 Cells

In an initial screen using the search-programs at the websites of the siRNA companies Dharmacon and Oligoengine, three sequences were selected - 1151, 1524 and 2348. (Numbers correspond to the position of the starting nucleotide in DEP-1 mRNA). Another published sequence (441) for DEP-1 suppression (Lampugnani, Zanetti et al. 2003) was additionally included. All these sequences were successfully cloned into pSUPER.retro.puro in order to facilitate retrovirus mediated infection of the target cells. As shown in Fig. 3.23, in transient transfections, all the four constructs had an inhibitory effect on DEP-1 expression when compared to the vector controls, albeit with different efficiencies.

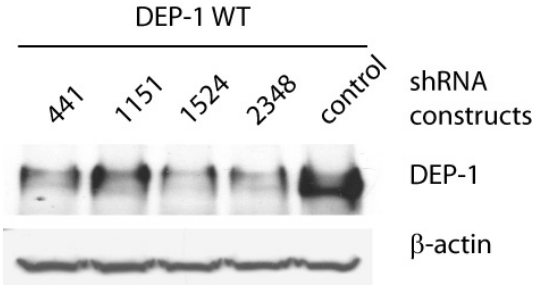


Fig. 3.23

Fig. 3.23 shRNA mediated suppression of DEP-1 – testing of different shRNA constructs.

HEK 293 cells were transiently co-transfected with DEP-1 WT construct and different shRNA constructs (numbered according to the first nucleotide in the targeted sequence of the mRNA) or a non-targeting control. 4 days after transfection, cells were lysed and lysates were immunoblotted to detect the remaining DEP-1 levels. Loading controls were performed by immunoblotting of lysates against β-actin.

Thus, all the four designed constructs showed some level of suppression against their non-targeting controls. However, only one of them, construct 441 turned out to be useful in our further studies.

3.4.2. Stable Suppression of Endogenous DEP-1 in HT 29 Cells

Since the extent of suppression was very efficient with the construct 441, this construct was used for creating stably suppressed cell lines. Moreover, the construct was based on a retroviral system; hence we chose to use the retroviral transduction for achieving better

efficiency and stability in these cell pools. Retroviruses were produced by transfection of a packaging cell line (Phoenix). As target cells, we chose the HT 29 cell line. These cells contain significant levels of DEP-1 (see Fig. 3.1a) and revealed intact density-dependent regulation (see Fig. 3.2). The HT 29 cells were infected with the produced retroviruses, thus facilitating stable integration of the shRNA construct into the genome. Since the extent of suppression was not satisfactory after one round of retroviral infection, a similar second round of infection was performed. From Fig. 3.24, it is clear that DEP-1 can be stably suppressed using such an approach, whereas the control cell line which contains the non-targeting sequence had unaltered DEP-1 expression.

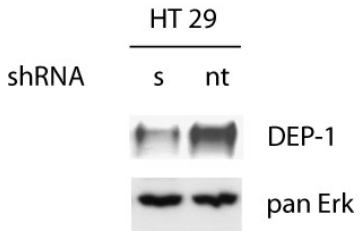


Fig. 3.24

Fig. 3.24 Suppression of endogenous DEP-1 in HT 29 cells by shRNA.

Construct 441 (s) and the non-targeting control (nt) were used to produce the retrovirus from the phoenix packaging cell line. Stable cell pools of HT 29 were generated after retroviral transduction with the resulting virus. IP of lysates were performed and the efficiency of DEP-1 protein suppression is shown by immunoblotting. Loading controls were performed by immunoblotting of lysates for total Erk1/2 levels.

Thus, using the construct 441, endogenous DEP-1 can be stably and efficiently suppressed in HT 29 cell line.

3.4.3. Proliferation Assay

Since the re-expression of DEP-1 WT reduced the proliferation of the SW 480 cells, it was obvious to speculate that the suppression of DEP-1 might result in a growth advantage. Proliferation assays were carried out using a fluorescent nuclear dye DAPI, which binds the DNA. As shown in Fig. 3.25, it is clear that the HT 29 cells with suppressed DEP-1 expression had a growth advantage compared to corresponding control cells, particularly under conditions of reduced serum (2% FBS), confirming the antiproliferative function of this PTP.

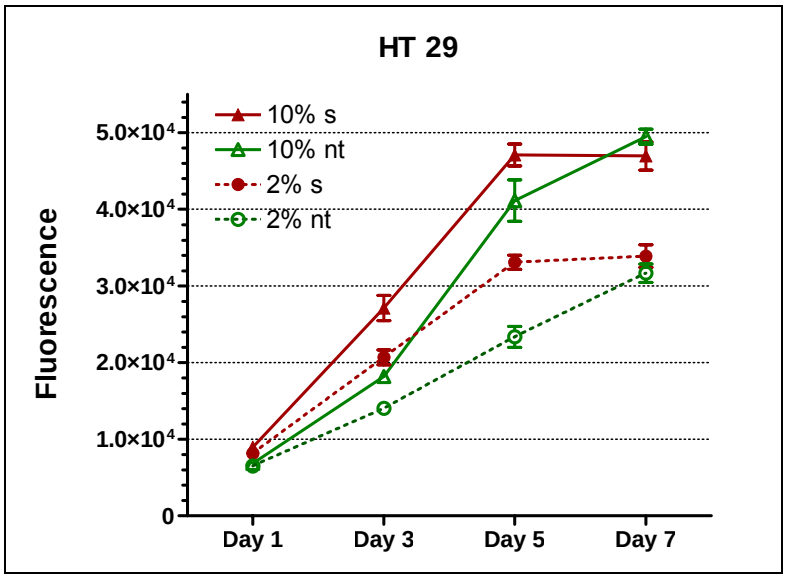


Fig. 3.25

Fig. 3.25 Enhanced proliferation of HT 29 cells upon suppression of endogenous DEP-1 by shRNA.

HT 29 cell pools with downregulated DEP-1 expression (s) or a corresponding control pool (nt) were analysed for cell growth. 8,000 cells/well in 96 well plates were seeded at different concentrations of fetal bovine serum (2% or 10%, as indicated). On the corresponding days, cells were fixed with methanol followed by staining with a nuclear dye 4',6-Diamidino-2-phenylindole-2HCl (DAPI). The fluorescence emitted by the bound DAPI was taken as a measure of the cell amounts.

These data show that upon suppression of endogenous DEP-1 in HT 29, the cells acquire a growth advantage emphasizing the anti-proliferative effect of DEP-1.

3.4.4. Immunoblot Analysis of Phospho-protein Molecules

The re-expression of DEP-1 had revealed both hypo- and hyper-phosphorylation of various phospho-proteins involved in signaling. Hence, we wanted to know whether the suppression of DEP-1 can also affect on the same molecules. Various signaling molecules were, however, not affected by DEP-1 suppression. These include the phospho-paxillin, FAK and Erk (not shown). We cannot say at this point whether this discrepancy to the data in DEP-1 re-expressing SW 480 cells is due to the different cellular backgrounds or an issue of DEP-1 levels. However, an effect was reproducibly seen on Akt phosphorylation upon DEP-1 suppression. From Fig. 3.26, it is clear that the serum-stimulated phosphorylation of Akt is elevated in the cell lines with suppressed DEP-1 expression when compared to their non-targeting controls.

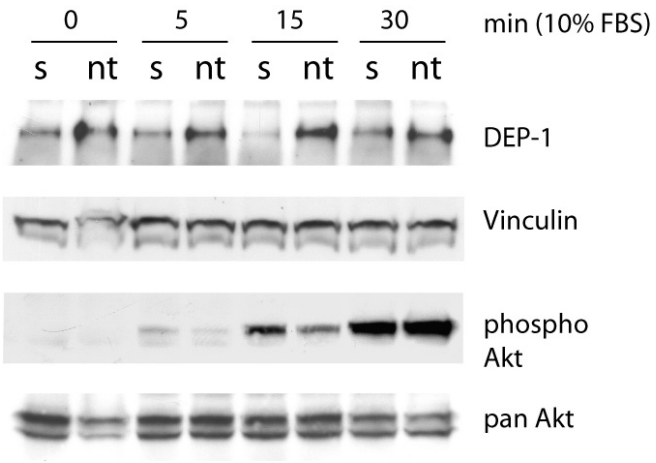


Fig. 3.26

Fig. 3.26 Hyper-phosphorylation of Akt upon suppression of DEP-1.

HT 29 cell pools with downregulated DEP-1 expression (s) or a corresponding control pool (nt) were seeded in 6.0 cm dishes. Cells were starved overnight and stimulated with 10% FBS for the indicated time points. Lysates were probed to detect the phosphorylation status of Akt. Total Akt levels in the same samples were also detected. The membrane was also probed with DEP-1 antibody to detect the extent of suppression and loading controls were shown with vinculin.

Thus, suppression of endogenous DEP-1 in HT 29 leads to hyper-phosphorylation of Akt in contrast to hypo-phosphorylation of Akt upon re-expression of DEP-1 in SW 480.

3.4.5. RTK Array Analysis

Since the phosphorylation of Akt is reduced in DEP-1 re-expressing cell lines and an inverse effect was observed upon DEP-1 suppression, we were interested in identifying the upstream kinase (potentially some RTK) that signals to Akt phosphorylation and may be the target of DEP-1. Hence we employed a RTK array (R&D) that allows the simultaneous detection of the phosphorylation status of 42 different RTKs. The cells from the DEP-1 suppressed and non-targeted cells were either left unstimulated or were stimulated with FBS for 15 min. They were then lysed and analysed with the RTK array. As shown in Fig. 3.27, a few RTKs including insulin receptor (box 2 coded in red) and insulin-like growth factor-1-receptor (IGF-1R, box 3 coded in pink) were hyper-phosphorylated due to stimulation with 10% FBS. There was obviously no elevated phosphorylation of any RTK in the cells with suppressed DEP-1. Surprisingly, HGFR (box-1 shown in blue) was hyper-phosphorylated in the non-targeting pools irrespective

of stimulation. Interestingly in the same experiment, robust Akt hyper-phosphorylation was seen in cells with suppressed DEP-1 expression. Another HT 29 cell line, where DEP-1 WT was over-expressed, was also analysed, but again no obvious changes were observed (not shown).

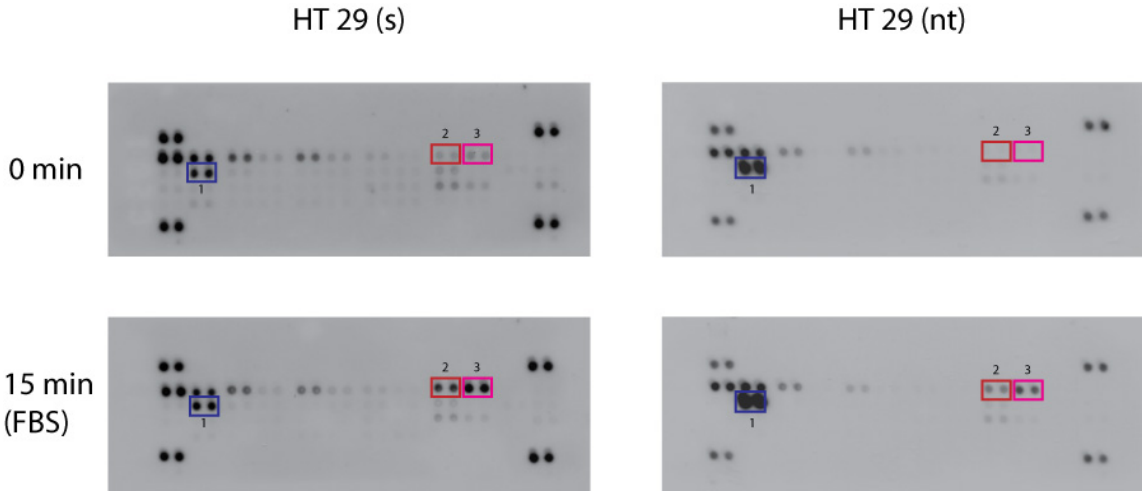


Fig. 3.27

Fig. 3.27 RTK array analysis of the DEP-1 suppressed (s) and non-targeting (nt) pools.

HT 29 cell pools with downregulated DEP-1 expression (HT 29 - s) or a corresponding non-targeting control pool (HT 29 - nt) were seeded in 6.0 cm dishes. Cells were starved overnight and were either left unstimulated (top panel) or stimulated with 10% FBS for 15 min (lower panel). Lysates were probed with an antibody array as described by the manufacturer. The resulting chemiluminiscent signals were scored on X-ray films. Box number 1 coded blue denotes phosphorylation of HGFR. Box number 2 coded red and box number 3 coded pink denotes the phosphorylation of insulin receptor and insulin-like growth factor-1- receptor respectively.

These data reveals that the analysed RTKs are not the prime targets of DEP-1 upstream of Akt under the conditions analysed.

3.5. Tumor Suppressor DEP-1 and its Relation to Nutrient Components

The colon is constantly exposed to nutrients. Epidemiological studies have shown that colorectal cancer incidence could be significantly modulated by dietary intake of flavonoids as part of fruits and vegetables (Terry, Giovannucci et al. 2001). Apples are a significant part of the Western diet, and they are a major source of flavonoids. Various flavonoid compounds have been found to possess a range of cancer preventive activities

including prevention of oxidative DNA damage, inhibition of carcinogen activation, induction of carcinogen detoxifying systems, interaction with cellular signaling pathways and modulation of gene expression controlling proliferation, differentiation and apoptosis of cancer cells (Barth, Fahndrich et al. 2005). Another important nutrient of dietary origin is butyrate. Human colonic bacteria ferment oligo- and polysaccharides to short chain fatty acids (SCFA), mainly acetate, propionate and butyrate. Butyrate has been suggested to play a central role in reducing cancer risks by regulating differentiation, inducing apoptosis and inhibiting growth of tumor cells *in vitro* (Hague and Paraskeva 1995). Tea is one of the most popular beverages consumed worldwide. Interest in the beneficial effects of green tea has led to investigations on activities by its main catechin - epigallocatechin-3-gallate (EGCG). This antioxidative compound could contribute to cancer chemoprevention by acting antigenotoxic (Glei and Pool-Zobel 2006).

3.5.1. Upregulation of DEP-1 by Different Nutrient Components

We speculated that some of the beneficial effects of certain nutrients towards colorectal cancer might be mediated through DEP-1 upregulation. We tested different nutrient components such as a polyphenol extract from apple juice (AE), butyrate (But) and an extract from green tea (GT) for their effects on DEP-1 levles in colon epithelial cell lines. From Fig. 3.28a, it is clear that in the case of HT 29 and CaCo2 cells, DEP-1 expression can be upregulated, upon 72 h treatment with butyrate or green tea, whereas AE had no effect on DEP-1 levels in these cells. We also tested an adenoma cell line LT 97. In these cells all the nutrients tested upregulated DEP-1 expression (Fig. 3.28a). One of the nutrients AE was also tested in different concentrations which again showed a clear dose-dependent upregulation of DEP-1 expression (Fig. 3.28b).

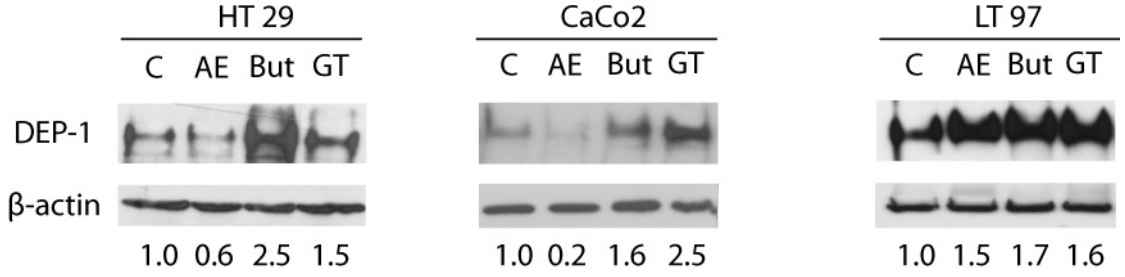


Fig. 3.28a

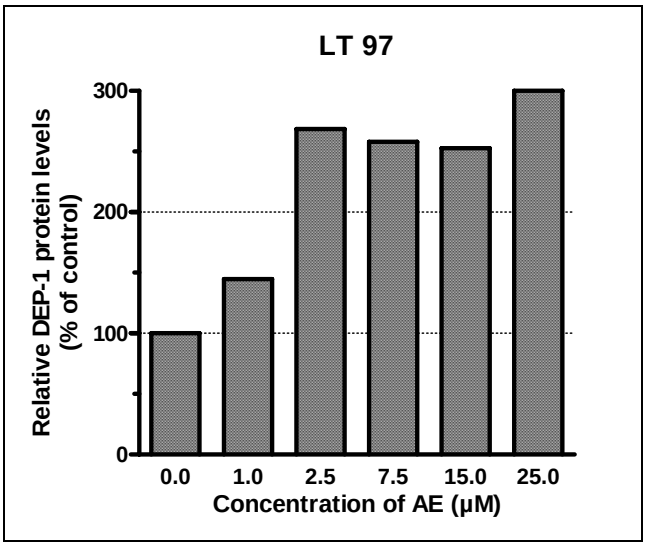


Fig. 3.28b

Fig. 3.28 Upregulation of DEP-1 expression by nutrient components in colon epithelial cells.

Fig. 3.28a Subconfluent cultures of HT 29, CaCo2 and LT 97 cells were left untreated (control, C), or were treated for 72 h with 2 mM sodium butyrate (But), 25 µM phloridzin equivalents of apple polyphenol extract (AE) or 20 µM epigallocatechine-3-gallate equivalents of Green Tea (GT). The cells were lysed and IP was performed to detect DEP-1 expression. Lysate aliquots were analysed for actin levels as input control. The shown experiment is representative of four with consistent results. Numbers under the lanes indicate relative amounts determined by densitometry of the DEP-1 signal.

Fig. 3.28b Subconfluent cultures of LT 97 adenoma cells were treated with apple polyphenol extract (concentrations equivalent to 0, 1, 2.5, 15 or 25 µM phloridzin), for 72 h and DEP-1 expression was determined by IP. The resulting blots were scanned, normalized to vinculin levels and a densitometric plot is shown.

These data reveal that DEP-1 expression can be upregulated in all the cell lines analysed by most of the tested nutrient components. Furthermore, polyphenol extract from apple can upregulate DEP-1 expression in a dose-dependent manner. The anti-proliferative effects of these nutrients (Pool-Zobel, Selvaraju et al. 2005; Shimizu, Deguchi et al. 2005; Veeriah, Kautenburger et al. 2006) could be linked to DEP-1 upregulation. However, this may not be the only mechanism, as the nutrient treatment of the DEP-1 depleted HT 29 cells also exhibited growth inhibition (not shown).

3.5.2. Induction of DEP-1 Occurs at the Transcriptional Level

Western blot analysis revealed the upregulation of DEP-1 by treatment with different nutrient components in LT 97 cells. In order to know whether the upregulation of DEP-1

was at the transcriptional or at the translational level, mRNA quantification of DEP-1 using Real Time PCR was performed. We observed an induction of DEP-1 mRNA in LT 97 adenoma cells by all the three tested nutrient components to a similar extent as observed at the protein level (Fig. 3.29).

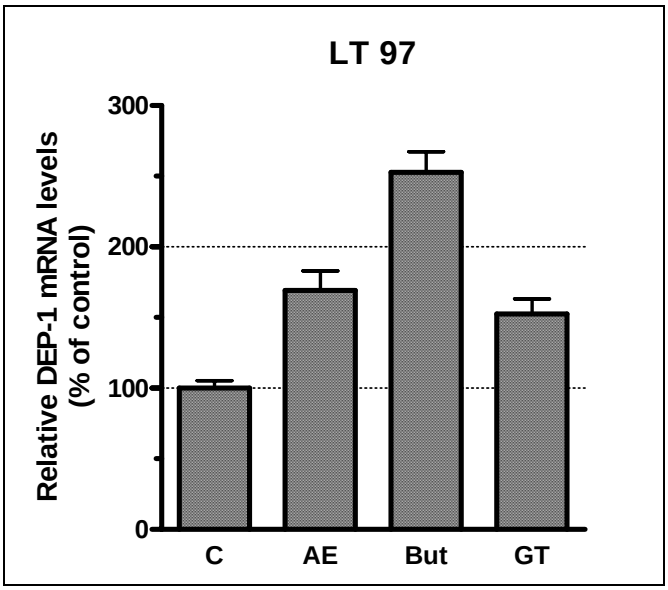


Fig. 3.29

Fig. 3.29 Upregulation of DEP-1 mRNA upon treatment with protective nutrients.

Subconfluent cultures were treated for 72 h as described in Fig. 3.28a Total RNA was extracted and subjected to real-time reverse transcriptase - polymerase chain reaction. Values were normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA detected in the same samples and were expressed relative to results for untreated cells (100%) (mean $\pm$ s.d., n=3).

Since the extent of upregulation obtained at the mRNA level correlates with the level of upregulation obtained at protein levels, we can conclude that upregulation of DEP-1 by these nutrient components is mainly transcriptional.

3.5.3. No Detectable Effect of Apple Juice on DEP-1 Transcript Levels In Vivo

Upon treatment with apple extract, DEP-1 is upregulated in adenoma cells but not in carcinoma cells. Since adenoma mirrors more closely the normal tissue, we wondered if an effect of apple could also be scored *in vivo*. We therefore tested whether intake of apple juice (AJ) can upregulate DEP-1 expression in the colonic mucosa of rats. Rats were fed either with apple juice (treatment) or water (control). One subgroup of animals

was also injected intraperitoneally either with a carcinogen di-methyl hydrazine (DMH) or sodium chloride (NaCl) as the control. All these animal experiments were performed at the Institute of Nutritional Physiology, Karlsruhe and the RNA from the colonic mucosa was kindly provided by Dr. S. W. Barth. As shown in Fig. 3.30, the mRNA levels of DEP-1 in these experimental animals varied strongly. The data failed to reveal any significant upregulation of DEP-1 mRNA.

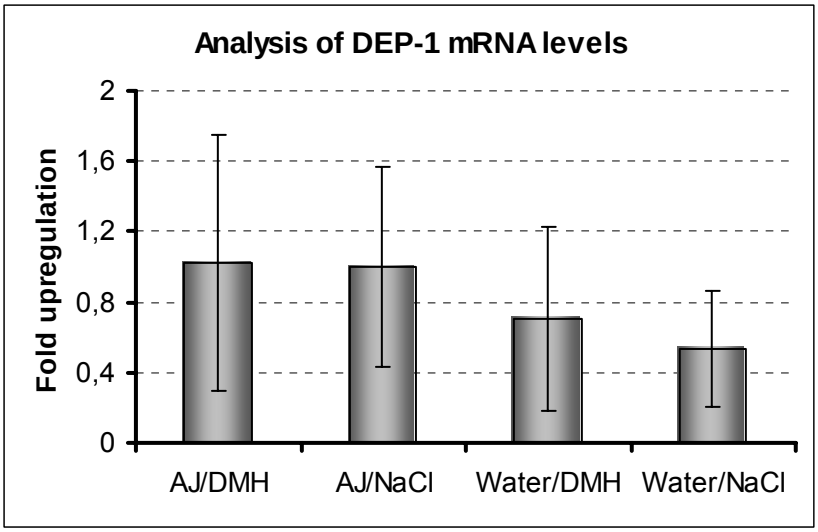


Fig. 3.30

Fig. 3.30 Apple juice drinking animals does not exhibit significant DEP-1 upregulation in their colonic mucosa.

Rats were fed either with apple juice (AJ) or water. Two sub-groups of animals were also injected intraperitoneally either with the carcinogen (DMH) or sodium chloride (NaCl) as control. The colonic mucosa of the animals was scraped off and total RNA was isolated and kindly provided by Dr. S. W. Barth. cDNA was synthesized from these RNA samples, and finally subjected to real time PCR to detect the mRNA level of DEP-1 (see Methods). Four animals in each group were used for these studies.

3.5.4. Induction of Apoptosis by Apple Polyphenol Extract in HT 29

In the literature (Hague and Paraskeva 1995; Wenzel, Kuntz et al. 2000), it has been suggested that various nutrients including butyrate and other flavonoids may also exert their beneficial effects by inducing apoptosis of colon carcinoma cells. As has been shown by others, the apple polyphenol extract has a cytostatic activity on HT 29 cells (Veeriah, Kautenburger et al. 2006) but the mechanistic basis for the cytostatic effect has been yet unknown. Apoptosis induction would be a possibility. Hence, we were curious to know whether the polyphenol extract of the apple juice (AE) can induce apoptosis.

Apoptosis was scored by the cleavage of poly (ADP-ribose) polymerase (PARP), which in HT 29 cells can be easily detected with an antibody. The antibody employed detects both the uncleaved (116 kDa) and cleaved (89 kDa) forms of PARP. As shown in Fig. 3.31, treatment of the HT 29 cells with AE can induce apoptosis in a dose and time dependent manner.

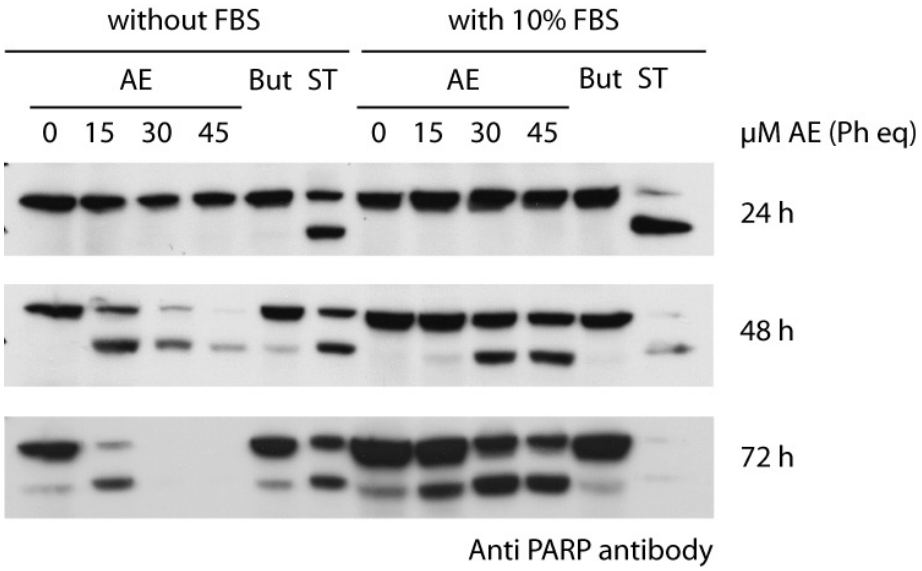


Fig. 3.31

Fig. 3.31 Induction of apoptosis by apple polyphenol extract (AE).

Sub-confluent HT 29 cells were either treated with apple polyphenol extract (AE) at concentrations equivalent to 0, 15, 30 and 45 µM phloridzin (Ph eq) or treated with 2 mM butyrate (But) or 1 µM staurosporine (ST) and checked for apoptosis induction. At the indicated time points (24 h, 48 h and 72 h) cells were lysed and lysates were immunoblotted to detect the cleavage of PARP. Apoptosis assay was performed both in the presence and in the absence of serum.

These data reveal that the polyphenol extract from apples can induce apoptosis in HT 29 cells and may at least be in part responsible for the observed cytostatic effect.

4. Discussion

Protein tyrosine phosphatases are key regulators of protein tyrosine phosphorylation. They have been implicated in a range of physiological processes, de-regulation of which can lead to various diseases including cancer. One of the transmembrane phosphatases, DEP-1 (Density Enhanced Phosphatase-1), has been proposed as a tumor suppressor in the case of glioma, mammary, pancreas and thyroid carcinomas. It has been mapped down to the colon cancer susceptibility locus (*Scc 1*) in mice (Ruivenkamp, van Wezel et al. 2002). Recently, it has been reported that loss of heterozygosity (LOH) on 11p11, in the region containing *PTPRJ*, is an early event in colon carcinogenesis (Luo, Shen et al. 2006). These and other data suggest DEP-1 as a candidate tumor suppressor for colon carcinogenesis. Nevertheless, the physiological role of DEP-1 in colon cells has largely remained ill-defined. Here we show that Density Enhanced Phosphatase-1 (DEP-1) negatively regulates proliferation and migration in colon cancer cells, and is associated with distinct cytoskeletal changes. Moreover, DEP-1 also exhibits a pro-apoptotic function and sensitizes the cells towards apoptosis. Furthermore, we were able to partly dissect the signaling mechanism of DEP-1 in these colon cancer cells.

4.1. Model System: Cell lines with inducible DEP-1 expression and shRNA mediated suppression

4.1.1. Inducible DEP-1 (WT or CS) expression

One approach to analyse the physiological role of any protein is to over-express or re-express the protein of interest in appropriate cellular backgrounds. Stable cell clones can be achieved either with constitutive expression or having the expression in an inducible manner. Constitutive over-expression has several disadvantages. First of all, if the protein is lethal or harmful for the viability of the host cells, it may be impossible to cultivate them. Moreover, the continuous presence of the protein can make the cell to adapt to certain phenotypes which may not be physiological. Hence, we decided to create stable cell clones with inducible DEP-1 expression. The particular cellular background is

another aspect for such studies. The analysis of endogenous DEP-1 expression (at protein level) in a set of colon cancer cell lines revealed the expression to be variable (Fig. 3.1a). Interestingly, the relatively abundant DEP-1 expression in the adenoma cell line LT 97 may reflect that this cell line resembles more closely the normal colon epithelium than cancer cells. The genetic constitution of the cell lines with respect to the possible absence of one allele of *PTPRJ* is not known. Abundant expression in some colon cancer cell lines such as HT 29 suggests, however, that these cell lines probably possess both alleles and, therefore, have unaltered DEP-1 levels. One of the cell lines, SW 480, had no detectable DEP-1 protein expression. However, RT-PCR analysis revealed the presence of DEP-1 mRNA in this cell line (Fig. 3.1b). This indicates the presence of at least one functional DEP-1 allele, despite the protein level being very low. It should be pointed out, that the RT-PCR analysis did not allow concluding on the mRNA levels. They may also be very low. Because of the absence of DEP-1 in SW 480, this cell line was chosen for our re-expression studies. To achieve inducible re-expression, the vector pNRTIS 21 was the preferred choice. The use of this vector has several advantages: The vector is based on a bi-cistronic system and has the gene of interest and the tetracycline-repressed transactivator (tTA) under the tTA-responsive promoter. The neomycin resistance gene is under a separate SV-40 promoter. This setup enables to select for resistant clones in the absence of any target gene expression. The expression of DEP-1 can be simply turned "off" or "on" either by just adding or removing anhydrotetracycline in the growth media respectively (popularly referred to as "Tet off" system), allowing a simple and stringent way of controlling gene expression. We have also employed both DEP-1 WT and a catalytically inactive mutant of DEP-1 (C1239S) in which the active site cysteine is mutated to a serine residue. This helped to differentiate the need for an active phosphatase against possible other effects of protein expression such as a potential scaffolding function. Using this inducible, bi-cistronic vector system, we have conditionally re-expressed the DEP-1 WT or CS variant into the DEP-1 negative cell line SW 480 (Fig. 3.4a). Moreover, when lysates were subjected to phosphatase assay, the DEP-1 WT expressing cells showed four-fold increased activity than their non-expressing counterparts. The catalytically inactive variant did not exhibit any activity in either of the expressing or non-expressing states (Fig. 3.4b). Once successful clones were established,

the cells were routinely cultured with Atc, devoid of any target gene expression. This prevents the cell from adapting to any phenotype due to the exogenous DNA. Whenever necessary, DEP-1 expression was easily induced by just culturing the cells in the absence of Atc for 3 - 4 days. This approach also overcomes the problem of clonal variation as the same clone is analyzed in both the expressing and non-expressing states for their resulting phenotypes.

Even though we created stable cell lines with inducible expression, induction of DEP-1 was gradually reduced in later passages (Fig. 3.5). There are several possible explanations for this phenomenon. Although the cultures were strictly maintained in the repressed state, a minimal level of foreign gene expression occurs from the vector pNRTIS 21. This is required to express a basal level of tTA which is needed to initiate transcription upon removal of Atc. DEP-1 is anti-proliferative (see Fig. 3.6) and hence the cultures which express low level of DEP-1 will have a growth disadvantage. This may have led to overgrowth of cells which have lost DEP-1 expression. However, the cell lines engineered to express the catalytically inactive DEP-1 CS variant, which had no growth differences, also gradually lost induction. Cellular background is another issue to be considered here. Segregation of cells with respect to DEP-1 expression may occur by silencing of the inducible promoter, a phenomenon frequently seen in cancer cells (Prof. Jürgen Behrens, personal communication).

4.1.2. shRNA mediated suppression

Another approach to study the physiological relevance of a protein is to knock out the corresponding gene or to suppress its expression in those cells that normally harbors it and to directly compare the biological outcomes with the results of re-expression. Previously, antisense oligonucleotides were used for this purpose which will bind to the target mRNA thereby blocking translation. In recent years, the suppression of protein expression by the use of small interfering RNAs (siRNAs) has been successfully employed for this purpose. Here we have used a shRNA which upon processing will produce a short 21 nucleotide (nt) siRNA. This will target the corresponding DEP-1 mRNA transcript thereby bringing down the protein levels. In order to have efficient and

stable integration of the shRNA vector into the host genome, a retroviral based transduction approach was employed. A non-targeting vector which contains sequences that do not target any of the known coding sequences was also included to exclude any irrelevant effects. The HT 29 cell line was chosen for this purpose as this cell line had relatively high levels of endogenous DEP-1 (Fig. 3.1a) and also revealed intact density dependent upregulation (Fig. 3.2). We have designed four shRNA constructs (see Methods) targeting different regions of the mRNA transcript. Initially, all these four constructs were tested in transient transfections by co-transfecting DEP-1 WT and shRNA constructs in HEK 293 cells. Since the pulse-chase experiment revealed the half-life of DEP-1 to be approximately around 13 hours (Fig. 3.3), the transfected cells were lysed 96 hours after transfections to allow for sufficient downregulation of the existing DEP-1 protein. All the four constructs showed some level of suppression (Fig. 3.23) and one of them was successfully employed for generating a stably suppressed HT 29 cell line. Even though the retroviral mediated transduction protocol was followed, it should be noted that different cell lines have different levels of susceptibility to the retroviral infection. With a single round of infection, only a weak suppression was achieved. But when two rounds of retroviral infections were performed, approximately 70-80% of endogenous DEP-1 was efficiently and stably suppressed (Fig. 3.24). Using these two cellular models (re-expression and suppression) we further investigated the role of DEP-1 in relation to its role in the colon epithelial cells.

4.2. Phenotypes of DEP-1 re-expression in colon cancer cells

4.2.1. DEP-1 negatively regulates proliferation

DEP-1 has been named “Density-Enhanced Phosphatase” as the levels of this phosphatase increase upon an increase in cell density. One of the first publications describing the cloning of DEP-1 also showed that at least in human embryonic fibroblasts (WI-38) and foreskin fibroblasts (AG1518), DEP-1 levels increase as cells approach confluence with respect to both the protein levels as well as the phosphatase activity. These data led the authors to propose that DEP-1 can participate in contact mediated

growth arrest (Ostman, Yang et al. 1994). In support of this, contact inhibition of VEGF-induced proliferation in endothelial cells has been shown to be mediated through DEP-1 by antagonizing VEGFR signaling (Lampugnani, Zanetti et al. 2003). Moreover, several studies have indicated that DEP-1 can antagonize signaling of several growth factor receptors including PDGFR, EGFR, VEGFR and HGFR in various cell lines. DEP-1 has also been shown to decrease proliferation in case of glioma (Massa, Barbieri et al. 2004), breast (Keane, Lowrey et al. 1996), thyroid (Iuliano, Trapasso et al. 2003), and pancreatic cancer models (Trapasso, Yendamuri et al. 2004). However, in the case of NIH 3T3 fibroblasts, controversial results have been obtained. Previous work in our lab has shown that inducible expression of DEP-1 had no effect on proliferation (Jandt, Denner et al. 2003). Another group has shown that DEP-1 can inhibit proliferation under similar conditions (Kellie, Craggs et al. 2004). Recently, a bivalent form of a monoclonal antibody against CD 148 (i.e., DEP-1) has been shown to inhibit endothelial cell growth, thereby negatively regulating angiogenesis (Takahashi, Takahashi et al. 2006). Consistent with most of the above findings, the re-expression of DEP-1 severely inhibited proliferation also in colon cancer cells (Fig. 3.6a). A more robust effect was seen when the proliferation assay was performed after DEP-1 expressing cells were enriched with the help of MACS (Fig. 3.6c). The re-expression of the catalytically inactive variant however did not have any effect on proliferation (Fig. 3.6b). These findings suggest that the intact catalytically active DEP-1 is necessary for exerting growth inhibition. To complement this hypothesis we also subjected the DEP-1 suppressed HT 29 cell lines to proliferation assays. Using this approach, we were able to show that the suppression of endogenous DEP-1 in HT 29 cells resulted in a growth advantage when compared to cells expressing non-targeting control siRNA and the effect was even more pronounced under conditions of reduced serum (Fig. 3.25). Taken together, these data clearly reveal the anti-proliferative effect of DEP-1 in colon epithelial cells.

Generally, the proliferative ability of any cell is correlated with the phosphorylation status of Erk. In case of thyroid cells, the expression of DEP-1 WT has been correlated with decreased Erk phosphorylation (Trapasso, Iuliano et al. 2000; Iuliano, Trapasso et al. 2003). Similar observations have also been reported in the case of fibroblasts

following PDGF stimulation (Jandt, Denner et al. 2003; Kellie, Craggs et al. 2004). Also in T cells, CD 148 (i.e., DEP-1) expression has been shown to downregulate Erk phosphorylation following stimulation with an anti-TCR monoclonal antibody (Baker, Majeti et al. 2001). In glioma cells, DEP-1 has been proposed to directly interact with and dephosphorylate Erk following bFGF stimulation (Massa, Barbieri et al. 2004). In contrast, DEP-1 re-expressing colon cells did not reveal any difference in Erk phosphorylation following serum stimulation for 30 min (Fig. 3.18). When stimulated with specific growth factors specific effects became apparent. In case of stimulation with LPA again no difference in Erk phosphorylation was observed (Fig. 3.20). However, when the cells were stimulated with HGF, a complete inhibiton of Erk phosphorylation, associated with dephosphorylation of HGFR was observed (Fig. 3.21). This antagonizing effect of DEP-1 on HGFR signaling may, under certain conditions, contribute to the observed anti-proliferative effect. Clearly, our data are not compatible with a direct effect of DEP-1 on Erk1/2 activity. Also, since DEP-1 inhibits growth in the presence of FBS and in the absence of any clear effect on serum stimulated Erk1/2 activities, the contribution of this pathway to growth inhibition is not clear. It should be noted that Erk1/2 activity has not been analysed under conditions of the continuous presence of FBS.

4.2.2. DEP-1 promotes apoptosis

Tumor suppressors such as p53, retinoblastoma protein or PTEN have been extensively studied and their tumor suppressive effect has been linked to reduced cell survival or enhanced apoptosis. Since DEP-1 is a putative tumor suppressor, we wondered whether re-expression of DEP-1 might play a role in apoptosis regulation of the cell. It has been described in the case of pancreatic carcinoma cells that the expression of DEP-1 disrupts the cell cycle and leads to apoptosis (Trapasso, Yendamuri et al. 2004). Moreover, a "sensitized" RNAi screen of all human kinase and phosphatase genes has revealed *PTPRJ*, the gene encoding DEP-1 as encoding a cell death promoting phosphatase in the presence of apoptosis-inducing cytotoxic compounds (MacKeigan, Murphy et al. 2005). Consistent with these findings, we showed that the re-expression of DEP-1 WT can sensitize the colon cancer cells to staurosporine-induced apoptosis in a dose-dependent

manner (Fig. 3.16a). The need for an active phosphatase was confirmed since the re-expression of the catalytically inactive variant failed to induce any apoptosis (Fig. 3.16b).

Similar as the phosphorylation status of Erk is correlated with cell proliferation, the survival or apoptosis rate is frequently regulated by Akt phosphorylation. Even though the apoptotic index of DEP-1 expressing cells has been scored in the case of pancreatic cancer cell lines, no correlation has been made with Akt phosphorylation. However, in the case of fibroblasts, Akt phosphorylation was shown to be suppressed in DEP-1 expressing cells following PDGF stimulation (Kellie, Craggs et al. 2004). In contrast, in endothelial cells, stimulation of CD 148 activity with a bivalent antibody did not seem to affect Akt phosphorylation (Takahashi, Takahashi et al. 2006). In our studies in the DEP-1 re-expressing SW 480 cells, in contrast to Erk phosphorylation, Akt phosphorylation was consistently downregulated (Fig. 3.18, Fig. 3.20 and Fig. 3.21). It should be noted that these cells exhibited a high level of the basal Akt phosphorylation. Furthermore, HT 29 cells with downregulated DEP-1 expression exhibited hyper-phosphorylation of Akt following serum stimulation (Fig. 3.26). Thus, taking together the re-expression and suppression studies, we were able to show consistent effects on Akt phosphorylation. These results clearly suggest a physiological relevance of the negative regulation of Akt phosphorylation by DEP-1 expression, which may relate to regulating apoptosis. Akt cannot be a direct target for DEP-1 as it is phosphorylated only on serine and threonine residues. DEP-1 being a transmembrane phosphatase, it is easy to speculate the involvement of certain RTKs regulating Akt phosphorylation as targets for DEP-1. However, our RTK array analysis failed to reveal downregulation of any of the 42 RTKs spotted onto the membrane (Fig. 3.27). Hence, we hypothesize the involvement of RTKs that are not included in the array, or other cytoplasmic tyrosine kinases as targets of DEP-1 mediating Akt phosphorylation.

4.2.3. DEP-1 negatively regulates migration with associated changes in actin cytoskeleton and focal adhesion molecules

4.2.3.1. DEP-1 negatively regulates migration

Density Enhanced Phosphatase (DEP-1) is a large transmembrane phosphatase with eight extracellular fibronectin repeats. The presence of these motifs has often been implicated in either mediating cell-cell contacts or cell matrix adhesion and migration. Similar transmembrane phosphatases such as those of the LAR-PTP family including LAR-PTP, PTPσ and PTPδ have been shown to affect migration in different cellular models (Serra-Pages, Kedersha et al. 1995; Elchebly, Wagner et al. 1999). One of the closest relatives of DEP-1, GLEPP1 (*PTPRO*) and its mouse ortholog PTPφ have been shown to induce rapid dephosphorylation of molecules involved in focal adhesions (Pixley, Lee et al. 2001). Moreover, previous data from our lab have indicated that inducible expression of DEP-1 inhibits the migration of fibroblasts (Jandt, Denner et al. 2003). Consistent with these findings, we were able to show that DEP-1 negatively regulates migration also in colon cancer cells. In wound healing assays, DEP-1 WT inhibited the migration by as much as 60% (Fig. 3.7). Since these assays were performed over a period of 24 hours, we cannot rule out that the inhibition of migration could have been partly due to the lack of proliferation of the DEP-1 re-expressing cells. Hence, we went on further to confirm our findings with a modified Boyden Chamber Assay (transwell assay) which was performed only for a period of six hours. Again the re-expression of DEP-1 inhibited the migration around 50% in these colon cancer cells (Fig. 3.8). Moreover, DEP-1 inhibited the migration induced not only with FBS but also the migration induced with LPA (Fig. 3.10). Even though the scatter factor HGF is a well established motogen for several cellular systems, HGF induced migration only weakly in the cell lines under investigation. Nevertheless, in wounding assays, cells migrated when stimulated with HGF. Again the re-expression of DEP-1 inhibited migration (Fig. 3.11). Since FBS, LPA and HGF induce migration by activating different receptor classes, this observation suggests the existence of common cellular targets downstream of these receptors that are affected by DEP-1 expression. Similar to proliferation, the re-expression of only the DEP-1 WT strongly inhibited migration whereas the C1239S mutant had no effect on

migration. This reveals that DEP-1 does not merely act as a scaffold. It rather mediates its effects by dephosphorylating yet unidentified substrates.

4.2.3.2. DEP-1 induces cytoskeletal changes

The migration of the cell is always associated with dynamic changes in their actin cytoskeleton. Migration involves actin remodeling at both the cell front and rear. Since the re-expression of DEP-1 strongly inhibited migration; it was interesting to see the associated cytoskeletal changes. In the case of fibroblasts, DEP-1 expressing cells had fewer actin containing microfilament bundles and a reduced density of vinculin and paxillin-containing adhesion plaques (Kellie, Craggs et al. 2004). These authors attributed these altered cytoskeletal changes to defective focal adhesion turnover, with a lack of activation of FAK. In contrast, staining of DEP-1 re-expressing colon cancer cells with phalloidin which binds filamentous actin revealed abundant cortical actin bundles lining the cell periphery. A uniform pattern of bundled actin can be seen beneath the cell membrane which is clearly absent in DEP-1 non-expressing cells (Fig. 3.13, Fig. 3.14). Moreover, staining of the cells with phospho-paxillin or vinculin revealed a characteristic re-distribution of these focal adhesion molecules (Fig. 3.15). In the case of DEP-1 re-expressing cells, these molecules are concentrated on certain regions of the membrane which may be the point of contacts or focal adhesions of the cell membrane towards the surrounding matrix. In the case of their non-expressing counterparts, vinculin and phospho-paxillin are not restricted to certain regions of the membrane. Instead they are distributed throughout the cell periphery.

Usually, migration involves the formation of small and broad lamellipodia or thin and long filopodia. These processes are initiated by the action of the small GTPases Rac and Cdc 42 respectively. Another small GTPase, namely Rho, is often implicated in the formation of stress fibres. Therefore, we explored the activation state of Rac and Rho in DEP-1 re-expressing cells. The pull down of the active fraction of small GTPases Rac failed to reveal any differences between DEP-1 expressing and non-expressing cells. In contrast, Rho pull down revealed a small upregulation of active Rho in DEP-1 expressing cells (Fig. 3.22). Due to technical difficulties, thereafter pull down of active Rho failed,

and so these effects were neither sufficiently confirmed nor could the analysis be performed for extended time periods. Still, the potential involvement of Rho is interesting and warrants further investigation. Rho mediates stress fibre formation and has been linked to both promoting and inhibiting migration. Some recent literature has indicated that Rho GTPase may play a role in inhibiting migration by antagonizing the action of Rac GTPase (Ohta, Hartwig et al. 2006). Potentially, activation of Rho in the DEP-1 expressing SW 480 cells could lead to the formation of stress fibres resulting in inhibition of migration. However, the involvement of other small GTPases or other signaling pathways cannot be ruled out. Local activation of Rho and Rac can be another possibility as well. One future prospect is to dissect the local activation of Rho (and Rac) either only at the leading or trailing edges. Taken together, DEP-1 re-expressing cells exhibit a distinct actin cytoskeleton associated with inhibition of migration. Actin reorganization could be mediated by the small GTPase Rho.

4.2.3.3. DEP-1 causes hyper-phosphorylation of paxillin and FAK with a complete dephosphorylation of PLC γ

As cellular migration involves a continuous formation and disassembly (turn over) of focal adhesion complexes, it is worthwhile to have a closer look at the molecules involved. Most of these molecules are regulated by tyrosyl phosphorylation. PLC γ has already a well established role in cellular migration by binding to various RTKs. Upon activation of the RTKs, PLC γ has been reported to be directly phosphorylated by the corresponding RTKs and thus being activated. Activated PLC γ hydrolyzes phospho-inositide-(4,5)-bisphosphoate (PIP_2) to diacyl glycerol (DAG) and inositol-triphosphate (IP_3) releasing gelsolin (an actin binding protein) from PIP_2. PIP_2 is usually present in the membrane in the absence of stimulation. The released gelsolin then severs established actin filaments while cofilin and profilin will nucleate it, resulting in enhanced migration of the cells (Wells and Grandis 2003). Analysis of the tyrosyl phosphorylation of PLC γ at $pTyr^{783}$ in DEP-1 expressing cells revealed a complete dephosphorylation irrespective of stimulation (Fig. 3.17a). Absence of the tyrosyl phosphorylation on PLC γ correlates well with the anti-migratory role of DEP-1.

c-Src kinase is a non-receptor tyrosine kinase that has long been known to be involved in cell-substratum adhesion. Knock out of c-Src affects the adhesive properties of the cells (Kaplan, Swedlow et al. 1995). The activity of c-Src is regulated by the phosphorylation status of two tyrosine sites: Tyr^{529} and Tyr^{418}. The carboxy-terminal Tyr^{529}, after its phosphorylation (usually by c-Src kinase abbreviated as Csk), interacts with the c-Src SH2 domain. As a consequence of this intramolecular binding, c-Src remains in an inactive conformation and does not exert any tyrosine kinase activity (Yeatman 2004). Several tyrosine phosphatases such as PTPα and PTP1B can activate c-Src by dephosphorylating phospho-tyrosine 529 (Zheng, Wang et al. 1992; Bjorge, Jakymiw et al. 2000), thereby counteracting the activity of Csk. Conversely, dephosphorylation of the second regulatory site of c-Src, Tyr^{418}, leads to inhibition of c-Src tyrosine kinase activity. Src mediated phosphorylation of paxillin and FAK has also been proposed to increase the adhesion and to inhibit the migration of cells. Moreover, r-PTPη, the rat homolog of DEP-1 has been shown to directly interact with and to dephosphorylate the c-Src inhibitory tyrosine residue (Tyr^{529}) both *in vitro* and in intact cells (Pera, Iuliano et al. 2005). Our results also show a slight decrease in phosphorylation of the inhibitory tyrosine residue (Tyr^{529}) associated with an increased phosphorylation at the autophosphorylation site (Tyr^{418}) in the case of DEP-1 expressing cells (Fig. 3.17c). This finding indicates that c-Src activity may be elevated upon DEP-1 re-expression. However, Src activity assays directly showing c-Src activation were not performed.

Focal Adhesion Kinase (FAK) is an intracellular tyrosine kinase and has been initially identified as a substrate of the viral Src oncoprotein. It has been found as a highly tyrosyl phosphorylated protein that localizes to integrin-enriched cell adhesion sites (Mitra, Hanson et al. 2005). FAK contains six tyrosyl phosphorylation sites. Tyr^{397} is the autophosphorylation site and has been shown to be phosphorylated in response to a variety of growth factors and integrin activation. All the other sites have been proposed to be phosphorylated by activated c-Src. The kinase domain has two phospho-tyrosines (Tyr^{576} and Tyr^{577}) both of which are not affected due to the re-expression of DEP-1 in these colon cells (Fig. 3.17b). This may indicate that the kinase activity of FAK is not altered due to re-expression of DEP-1, although again, this has not been directly tested.

However, other phospho-tyrosines of FAK (Tyr^{407}, Tyr^{861} and Tyr^{925}) showed hyper-phosphorylation in DEP-1 re-expressing cells (Fig. 3.17b). The activation of c-Src as demonstrated by the hyper-phosphorylation at Tyr^{418} may account for the site-specific hyper-phosphorylation of FAK. The tyrosine residue at 861 has been implicated in binding to p130cas (Lim, Han et al. 2004) which then binds to the adaptor protein Crk II resulting potentially in the activation of the small GTPase Rac (Cary, Han et al. 1998; Klemke, Leng et al. 1998). However, we did not observe any Rac activation in the DEP-1 re-expressing cells and hence the role of the phosphorylation of FAK at Tyr^{861} in this context is not clear. Tyr^{925} of FAK is also hyper-phosphorylated in DEP-1 re-expressing cells. It has been shown that Tyr^{925} of FAK binds to GRB2 adaptor protein and feeds to the Ras/MAPK cascade. Subsequently, Erk phosphorylation of MLCK can modulate focal contact dynamics resulting in enhanced motility of the cells. Even though FAK^{925} was hyper-phosphorylated, no difference in the phosphorylation status of Erk was observed in these DEP-1 expressing cells which may explain the hypo-migratory phenotype of these cells. Moreover, consistent with our findings (Fig. 3.17a and Fig. 3.17b), restoration of r-PTPη in thyroid cells led to enhanced tyrosyl phosphorylation of FAK and paxillin (Pera, Iuliano et al. 2005).

Paxillin is another focal adhesion protein that mediates the signals from the extracellular matrix to actin cytoskeleton and is regulated by two tyrosyl phosphorylation sites (Tyr^{31} andTyr^{118}) (Turner 2000). Some groups have reported paxillin to be phosphorylated by active c-Src, whereas others claim that Tyr^{118} is a principal phosphorylation site for activated FAK (Bellis, Miller et al. 1995). Various studies, including knock out studies indicate paxillin as a positive effector of cell migration. Similar to FAK, activated paxillin has also been proposed to bind to the adaptor protein Crk leading to the recruitment of Rac GEF (Dock 180) resulting in Rac activation (Schaller and Parsons 1995). Phosphorylated paxillin has also been shown to locally suppress RhoA activity by activating p190RhoGAP thereby playing a role in membrane spreading (Tsubouchi, Sakakura et al. 2002). All these reports implicate paxillin as a positive regulator of cellular migration. However, since there was no change in total Rac activation in DEP-1

re-expressing SW 480 cells, these well established pathways may not play any role in the migratory phenotype of these cells.

In contrast to the above quoted reports that the activation of FAK or paxillin promotes cell migration, a recent study in HeLa cells showed that the siRNA mediated knock-down of either FAK or paxillin resulted in enhanced migration on collagen coated plates (Yano, Mazaki et al. 2004). In a previous study, the same authors have shown that paxillinα and p130cas exert opposing effects and that paxillin can act as a negative regulator of cellular migration (Yano, Uchida et al. 2000). Local down regulation of Rac by paxillin and FAK was suggested as a mechanism for the anti-migratory phenotype (Schaller 2004). Again, local effects on Rac cannot be excluded based on our biochemical data. In conclusion, the signaling pathway analysis in the DEP-1 re-expressing cells is not easily interpretable in the light of the current knowledge on regulation of cell migration. More data, notably on local signaling events are required for a better understanding.

Another possibility to identify the critical steps in the anti-migratory action of DEP-1 is to analyse the cells with down regulated DEP-1 expression for the phosphorylation status of these discussed focal adhesion molecules. However, no significant differences in the phosphorylation status of either paxillin or FAK were observed. These results can be interpretated at least in two ways. One issue concerns the general morphology of the cells used for the re-expression (SW 480), or suppression (HT 29) studies. SW 480 cells are larger, spindle shaped and has a very short doubling time. On the other hand, HT 29 cells are much smaller in size and usually grow in clumps or islands. Hence these cells may employ different molecules or mechanisms for their cell matrix interactions, mediating adhesion and migration. One possibility to overcome this problem may be to employ DEP-1 suppression in CaCo2 cells, as the morphology of these cells resembles more closely to SW 480. A second issue can be linked to the levels of DEP-1 expression. During re-expression of DEP-1 in SW 480 cells, supraphysiological levels may be attained. As a result, DEP-1 is forced to dephosphorylate non-physiological substrates which may be responsible for these observed hyper-phosphorylation of paxillin and FAK. However, the alternative may also hold true. Since the siRNA mediated suppression was

not 100%, the remaining small amounts of DEP-1 may still be sufficient to perform its physiological role. Surely re-expression or suppression in additional cell lines will shed more light on the physiological targets of DEP-1 in cytoskeletal regulation.

4.2.4. Re-expression of DEP-1 antagonizes c-Met

HGF, popularly referred to as "scatter factor" acts by inducing phosphorylation of the RTK, c-Met (HGFR). In the case of starved cells, HGFR phosphorylation cannot be seen in both DEP-1 expressing and non-expressing cells. However after stimulation, phosphorylation was visible only in the case of DEP-1 non-expressing cells (Fig. 3.21). It should also be noted that the receptor levels (HGFR) appeared somewhat less in the case of DEP-1 expressing cells. After HGF stimulation, phosphorylation was not only indetectable for its receptor (HGFR), but also a complete blockade of downstream signaling events, notably the HGF-stimulated phosphorylation of Erk was observed. HGF is a so called "scatter factor" as the signaling by this molecule causes the cells to scatter or to migrate. HGF is also a potent mitogen as it has been shown to enhance proliferation and migration in HT 29 cells (Dignass, Lynch-Devaney et al. 1994). HGFR has also been shown to regulate the anti-apoptotic protein bcl-x(L) thereby inhibiting apoptosis (Kitamura, Kondo et al. 2000). Being an established motogen, it is no wonder that the HGF induced migration is believed to play an important role in the context of cancer metastasis. A recent study has shown that HGF inhibitors can block HGF-induced invasion of cancer cells (Parr and Jiang 2006). Hence antagonizing the action of this receptor HGFR may be very important in the context of tumor suppression. An initial substrate trapping approach (using the DA mutants) of DEP-1 in breast tumor cell lines revealed the possibility of HGFR as a substrate for DEP-1. The authors went on to show that DEP-1 can be immunoprecipitated with HGFR in over-expression studies using HEK 293 cells (Palka, Park et al. 2003). Consistent with their findings, re-expression of DEP-1 in colon cancer cells also inhibited HGFR signaling. At this point, special emphasis has to be laid on Erk phosphorylation. Following LPA or serum stimulation, no obvious difference in the phosphorylation status of Erk was observed (Fig. 3.18 and Fig. 3.20). In contrast, phosphorylation of Erk was completely undetectable following HGF stimulation (Fig. 3.21). This shows that DEP-1 can specifically antagonize the signals emanating

from this receptor (HGFR) which may be an important aspect of its tumor-suppressive activity.

4.2.5. DEP-1 does not play a role in cellular adhesion

Cellular migration requires a series of repetitive and integrated processes to produce coordinated cellular movements. It involves a constant turn-over of adhesion complexes in which it initiates the formation of new adhesion complexes at the cell front and has to dismantle them again at the cell rear. One possibility for the observed inhibition of cellular migration could be an enhanced adhesion of the cells to the matrix components. Moreover, various phosphatases such as PTPα, PTP1B, and PTP-PEST have been reported to alter the adhesive properties of the cell (Burridge, Sastry et al. 2006). The role of DEP-1 in relation to adhesion has been studied in the case of fibroblasts. Previous data from our group indicated that DEP-1 acts as a positive effector of cell-substrate adhesion (Jandt, Denner et al. 2003). In contrast, another group has shown that DEP-1 negatively regulates cell-substratum interactions, whereas enhancing cell-cell adhesion (Kellie, Craggs et al. 2004). In the case of malignant thyroid cells, the expression of DEP-1 has been implicated in enhanced cell-substratum adhesion (Pera, Iuliano et al. 2005) and this effect has been attributed to the dephosphorylation of the c-Src inhibitory tyrosine residue (Tyr^{529}). Moreover, during trypsinization of the DEP-1 expressing and non-expressing cells for our experiments, it always appeared that the DEP-1 expressing cells had a tendency to attach stronger to the substratum than their non-expressing counterparts. In contrast, the experimental results indicated that the re-expression of DEP-1 did not affect cell-matrix adhesion (Fig. 3.12). To assess possible changes, we have employed two different methods: the first one measured the rate at which the cell attached to the substratum, and the second one scored the detachment rate of the cell from the substratum. Perhaps, in this analysed SW 480 cell line, DEP-1 may not play any role in cell-matrix adhesion or the assays which we performed were not sensitive enough to score some subtle differences.

4.2.6. Lessons from Knock-out mice

There are currently two published reports on *Ptprj* knock out mice which have revealed contradictory findings. The initial report showed that knocking out *Ptprj* in mice resulted in embryonic lethality (Takahashi, Takahashi et al. 2003). These authors have replaced *Ptprj* with a mutant allele CD148$^{\Delta CyGFP}$, an enzymatically inactive DEP-1-GFP (Green Fluorescent Protein). The mutant allele also possessed the extracellular domain of *Ptprj* whereas the cytoplasmic part was replaced in-frame with a cDNA encoding the GFP. Mice bearing such a mutation died at mid-gestation due to vascularization failure with disorganized vascular structures and cardiac defects. In contrast, recently obtained viable *Ptprj* knock out mice have been created by targeting the exons 3, 4 and 5 of *Ptprj*, resulting in its complete deletion (Trapasso, Drusco et al. 2006). Analyses of these mice have revealed that *Ptprj* is dispensable for growth and development. Moreover, *Ptprj* gene inactivation did not predispose the mice to spontaneous tumorigenesis. One possibility for the controversial findings could be that, in the initial knock out mice, the extracellular domain of Ptprj is still present and only the cytoplasmic domain is replaced by GFP. This suggests that the extracellular domain of Ptprj might act as a functional ligand able to block a pathway responsible for the correct assembly of endothelial cells in the generation of normal vessels resulting in the lethal phenotype.

Given the existing body of evidence suggesting a tumor suppressive role of DEP-1 in different cancers including our own results, it is quite surprising that no spontaneous tumorigenesis was detectable in the *Ptprj*$^{-/-}$ mice. Since most of these previous data were obtained in established carcinoma cells (likely to have more than one genetic lesion) the effect of DEP-1 may depend on the status of other genes. It is possible that *Ptprj* loss co-operates with additional genetic lesions in cancerogenesis. One possibility to test this hypothesis is by crossing these viable KO mice with other genetically modified tumor prone mice. This approach has recently uncovered a tumor-suppressive capacity of PTP 1B (Dube, Bourdeau et al. 2005).

4.3. Upregulation of DEP-1 by protective nutrients

4.3.1. Apple polyphenols upregulate DEP-1 and induce apoptosis

Normal epithelial cells in the colon are constantly exposed to dietary components and their fermentation products. The incidence of colon cancer has often been correlated with the lifestyle and particularly to food intake. Intake of refined grain, red meat, processed meat and alcohol has been correlated with increased risk of colorectal cancer incidence. On the other hand, consumption of whole grain, raw and cooked vegetables, citrus and other fruits, green tea and red wine has been shown to reduce the risk of cancer incidence (Levi, Pasche et al. 1999). However, the underlying mechanisms are only partially characterized. Apples are a widely consumed, rich source of phytochemicals and epidemiological studies have linked the consumption of apples with the reduced risk of some cancers, cardiovascular disease, asthma and diabetes (Aprikian, Busserolles et al. 2002; Liu and Sun 2003). Apples have been found to have very strong antioxidant activity, inhibiting cancer cell proliferation, decreasing lipid oxidation and lowering cholesterol levels (Boyer and Liu 2004). Many of the beneficial effects of apple intake are believed to be caused by apple polyphenols, including quercetin, catechin, phloridzin and chlorogenic acid, all of which are strong antioxidants (Lu and Foo 1997). In cell culture studies, these flavonoids have been implicated in a range of processes including inhibition of cancer cell proliferation, induction of apoptosis, prevention of oxidative DNA damage and induction of carcinogen detoxifying enzymes. Furthermore, three independent groups have shown that apple intake by rats has a strong antiproliferative effect on carcinogen-induced lesions in the colon epithelium (Barth, Fahndrich et al. 2005; Gosse, Guyot et al. 2005; Graziani, D'Argenio et al. 2005). Using cDNA microarrays, a recent study has shown that an apple polyphenol extract induces several genes involved in drug metabolism thereby modulating the toxicological defense of various carcinogens against colon cancer cells (Veeriah, Kautenburger et al. 2006). In accordance with the upregulation of several beneficial genes by the apple polyphenol extract, the expression of DEP-1 is also upregulated upon treatment, at least in the tested adenoma cell line LT 97 (Fig. 3.28a). Our findings are consistent with the data obtained in a custom-designed expression array (Veeriah et al., unpublished data). While two

tested carcinoma cell lines failed to reveal any upregulation of DEP-1 expression, the adenoma cell line even exhibited a dose-dependent upregulation of DEP-1 with apple polyphenol extract (Fig. 3.28b). We further investigated whether this upregulation of DEP-1 can also be seen *in vivo*. In a study to assess the chemopreventive properties of apple juice, rats were fed either with the apple juice or water as the control. One group of rats was also injected with the carcinogen DMH, whereas the control group received only sodium chloride (see Methods and Results for more details). Analysis of the RNA samples from apple juice drinking animals revealed only a slight trend but failed to show any significant upregulation (Fig. 3.30). This result could have been caused by different reasons. Either DEP-1 may not be significantly upregulated under *in vivo* conditions. Alternatively, and more likely, the negative result might be attributed to the sample preparation. In the rats, only the luminal parts of colon epithelial cells are exposed to the apple juice. DEP-1 regulation may occur only in certain components, such as epithelium in the crypts. RNA had been obtained by scraping off the mucosa for the analysis. This preparation may very well contain underlying cells or tissues that were not exposed to the apple juices, thereby changes in expression may have remained undetected. A solution to this problem could be the microdissection of tissue before expression analysis.

Dietary flavonoids and polyphenols have been shown not only to inhibit proliferation of cancer cells (cytostatic effect) but also to promote apoptosis in colon cancer cells (Wenzel, Kuntz et al. 2000). Hence, we wondered whether the complex apple polyphenol extract could do so. In accordance with the published literature, we can clearly show that the flavonoids present in the apple extract can also induce apoptosis in a dose- and time-dependent manner as depicted by the PARP cleavage (Fig. 3.31). Thus, from our studies it could be suggested that the apple polyphenols exert their beneficial effects not only by upregulating the tumor suppressor DEP-1, but also by inducing apoptosis.

4.3.2. Upregulation of DEP-1 by butyrate and green tea components

The beneficial effects of short chain fatty acids are also well established, in particular for butyrate. Butyrate is a non-specific inhibitor of histone deacetylases, thereby regulating transcription (Hassig, Tong et al. 1997). Butyrate is known for its anti-proliferative

activity on colon cancer cells in which it also induces apoptosis, whereas it acts as a survival factor and a nutrient in non-transformed cells as well (Pool-Zobel, Selvaraju et al. 2005). Butyrate has earlier been shown to enhance DEP-1 expression and to induce differentiation in mammary carcinoma cell lines such as ZR75 and SKBr-3 (Keane, Lowrey et al. 1996). Consistent with the effect on mammary carcinoma lines, butyrate also upregulated DEP-1 expression in all the colon cancer cells tested, albeit to a different extent (Fig. 3.28a). Thus, in addition to apple polyphenols, butyrate as a fermentation product may also mediate an anti-proliferative effect by elevated DEP-1.

Epigallocatechine-3-gallate (EGCG), a major component of green tea, and related constituents have various anticancer effects, including a marked antioxidant potential (Ames, Gold et al. 1995), induction of apoptosis (Weinreb, Mandel et al. 2003) and likewise inhibition of proliferation of human colon cancer cells (Shimizu, Deguchi et al. 2005). Similar to butyrate, treatment with green tea resulted again in upregulation of DEP-1 expression in all the cell lines analysed. Thus, green tea may also exert a protective role with respect to colon carcinogenesis by upregulating DEP-1 expression.

The induction of DEP-1 by the various dietary components cannot be attributed to increases in cell density, as all components either inhibited cell growth (butyrate and apple polyphenols) or had little influence on cell proliferation (green tea). We were also interested in understanding whether the upregulation of DEP-1 expression is either transcriptional or related to altered protein stability. Analysis of the mRNA from the adenoma cell line LT 97 treated with all three tested nutrient components revealed an upregulation of DEP-1 mRNA to a similar extent as observed at the protein level. This finding suggests that transcriptional regulation is, at least in part, the underlying mechanism of DEP-1 induction and is in line with current concepts of protective nutrient activity. Transcriptional regulation of the detoxifying enzymes such as Glutathione-S-Transferases (GSTs) by various dietary components is well established. It often involves an antioxidant-responsive response element (ARE) in the promoter of affected genes and the transcription factor nuclear factor E2-related factor 2 (Nrf2), which is bound to the Kelch-like ECH associated protein 1 (Keap1) in the cytoplasm. Antioxidants may disrupt

the Keap–Nrf2 complex, allowing Nrf2 to translocate to the nucleus and mediate expression of several GSTs via interaction with the ARE (Pool-Zobel, Veeriah et al. 2005). Similar transcriptional mechanisms may also hold true for DEP-1 upregulation. DEP-1 induction by nutrients may additionally also involve effects at the protein level, such as on protein stability. Induction of DEP-1 is, however, not the only mechanism of growth inhibition by butyrate or apple polyphenols, as shRNA-mediated depletion of DEP-1 did not render HT 29 cells refractory to growth inhibition by these agents. To our knowledge, this is the first report where the effects of protective nutrients are attributed to the upregulation of a tumor suppressor. This suggests an upregulation of DEP-1 as a previously unrecognized aspect of chemoprevention by dietary compounds.

5. Conclusion and Outlook

5.1. Conclusion

In conclusion, our data support that DEP-1 may be a tumor suppressor protein in colon cancer:

a) The expression of DEP-1 is partially or completely reduced in colon cancer cell lines
b) Its re-expression hinders cell proliferation and furthers cell death.
c) DEP-1 inhibits cell migration and causes hyper-phosphorylation of paxillin and FAK.
d) DEP-1 impairs activation of the Erk pathway upon stimulation with HGF.
e) DEP-1 is also upregulated upon treatment with various protective nutrients and thus may form one of the missing links between nutrients and cancer prevention.

Based on our data, we propose the following model for the mode of action of DEP-1. (Fig. 5.1). The transmembrane phosphatase DEP-1 can antagonize RTKs by directly dephosphorylating them, thereby modulating proliferation and apoptosis. DEP-1, by activating c-Src, causes hyper-phosphorylation of paxillin and FAK. Together with activating the small GTPase Rho, it induces severe cytoskeletal changes leading to inhibition of migration.

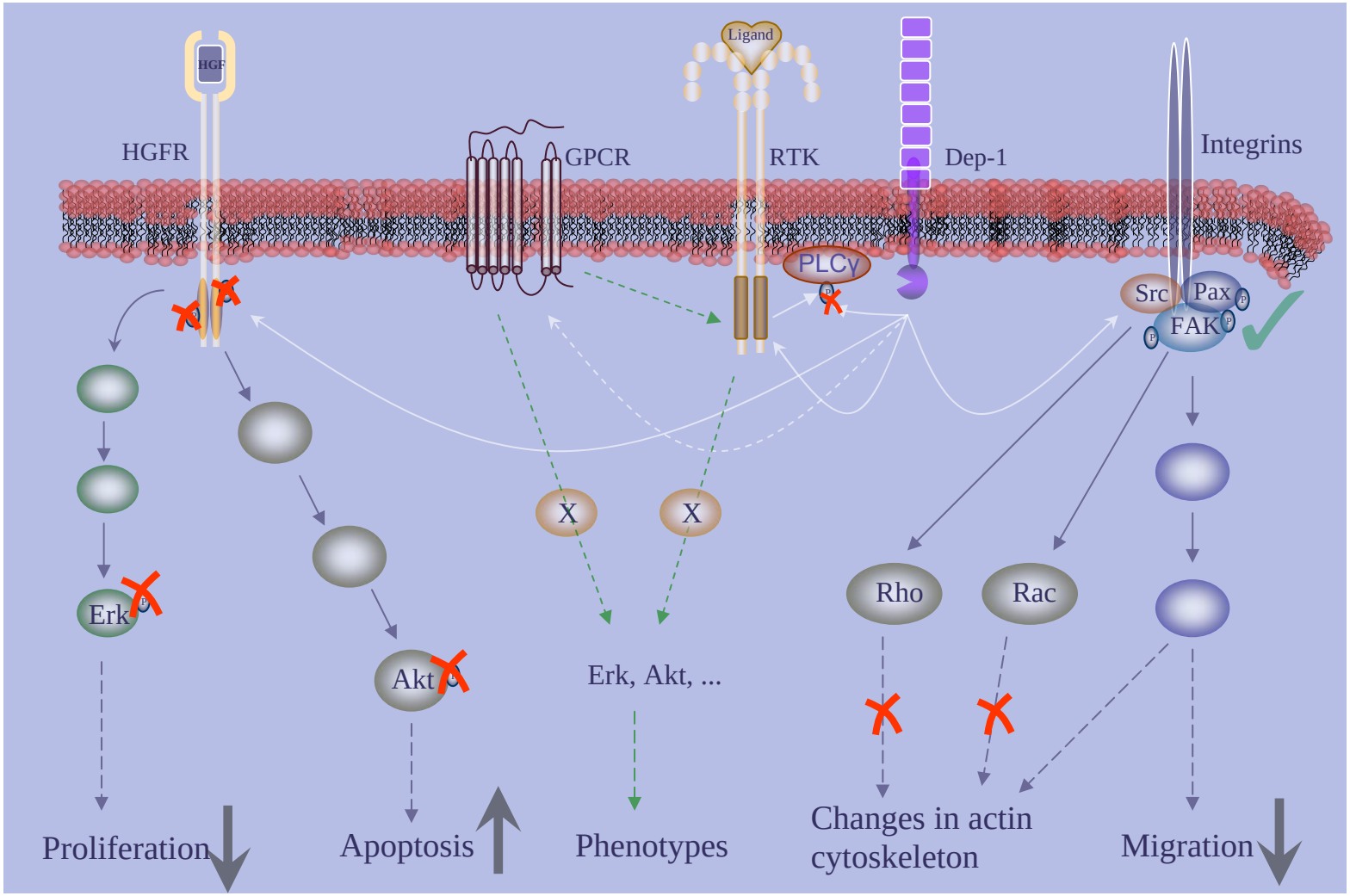


Fig. 5.1 Proposed model for the tumor suppressive action of DEP-1 in colon epithelial cells

5.2. Outlook

Even though our work has shed some light on the role of DEP-1 in colon epithelial cells, it also opens the door for a number of questions.

- Identification of DEP-1 substrates acting upstream of Akt.
- Completing the signaling chains responsible for the anti-migratory phenotype of DEP-1: Assay of Src and FAK tyrosine kinase activity, local changes of the small GTPases Rac and Rho.
- Follow up on the role of DEP-1 on HGF stimulated Erk downregulation.
- *In vivo* studies in nude mice: Transplantation of inducible DEP-1 expressing SW 480 cells into nude mice and assessing the transformation potential *in vivo*.
- Refinement of expression analysis in colon epithelial cells (microdissection)
- Identification of relevant polyphenols for DEP-1 upregulation.

6. Appendix

6.1 References

Adams, G. P. and L. M. Weiner (2005). "Monoclonal antibody therapy of cancer." Nat Biotechnol **23**(9): 1147-57.

Alonso, A., J. Sasin, et al. (2004). "Protein tyrosine phosphatases in the human genome." Cell **117**(6): 699-711.

Ames, B. N., L. S. Gold, et al. (1995). "The causes and prevention of cancer." Proc Natl Acad Sci U S A **92**(12): 5258-65.

Andersen, J. N., O. H. Mortensen, et al. (2001). "Structural and evolutionary relationships among protein tyrosine phosphatase domains." Mol Cell Biol **21**(21): 7117-36.

Angers-Loustau, A., J. F. Cote, et al. (1999). "Roles of protein tyrosine phosphatases in cell migration and adhesion." Biochem Cell Biol **77**(6): 493-505.

Aprikian, O., J. Busserolles, et al. (2002). "Lyophilized apple counteracts the development of hypercholesterolemia, oxidative stress, and renal dysfunction in obese Zucker rats." J Nutr **132**(7): 1969-76.

Araki, T., H. Nawa, et al. (2003). "Tyrosyl phosphorylation of Shp2 is required for normal ERK activation in response to some, but not all, growth factors." J Biol Chem **278**(43): 41677-84.

Argraves, W. S. and C. J. Drake (2005). "Genes critical to vasculogenesis as defined by systematic analysis of vascular defects in knockout mice." Anat Rec A Discov Mol Cell Evol Biol **286**(2): 875-84.

Autschbach, F., E. Palou, et al. (1999). "Expression of the membrane protein tyrosine phosphatase CD148 in human tissues." Tissue Antigens **54**(5): 485-98.

Baker, J. E., R. Majeti, et al. (2001). "Protein tyrosine phosphatase CD148-mediated inhibition of T-cell receptor signal transduction is associated with reduced LAT and phospholipase Cγ1 phosphorylation." Mol Cell Biol **21**(7): 2393-403.

Barth, S. W., C. Fahndrich, et al. (2005). "Cloudy apple juice decreases DNA damage, hyperproliferation and aberrant crypt foci development in the distal colon of DMH-initiated rats." Carcinogenesis **26**(8): 1414-21.

Bellis, S. L., J. T. Miller, et al. (1995). "Characterization of tyrosine phosphorylation of paxillin *in vitro* by focal adhesion kinase." J Biol Chem **270**(29): 17437-41.

Bentires-Alj, M., J. G. Paez, et al. (2004). "Activating mutations of the noonan syndrome-associated SHP2/PTPN11 gene in human solid tumors and adult acute myelogenous leukemia." Cancer Res **64**(24): 8816-20.

Berset, T. A., E. F. Hoier, et al. (2005). "The *C. elegans* homolog of the mammalian tumor suppressor Dep-1/Scc1 inhibits EGFR signaling to regulate binary cell fate decisions." Genes Dev **19**(11): 1328-40.

Beyer-Sehlmeyer, G., M. Glei, et al. (2003). "Butyrate is only one of several growth inhibitors produced during gut flora-mediated fermentation of dietary fibre sources." Br J Nutr **90**(6): 1057-70.

Bjorge, J. D., A. Jakymiw, et al. (2000). "Selected glimpses into the activation and function of Src kinase." Oncogene **19**(49): 5620-35.

Blume-Jensen, P. and T. Hunter (2001). "Oncogenic kinase signalling." Nature **411**(6835): 355-65.

Boyer, J. and R. H. Liu (2004). "Apple phytochemicals and their health benefits." Nutr J **3**: 5.

Brady-Kalnay, S. M., A. J. Flint, et al. (1993). "Homophilic binding of PTP mu, a receptor-type protein tyrosine phosphatase, can mediate cell-cell aggregation." J Cell Biol **122**(4): 961-72.

Brazil, D. P. and B. A. Hemmings (2001). "Ten years of protein kinase B signalling: a hard Akt to follow." Trends Biochem Sci **26**(11): 657-64.

Burridge, K., S. K. Sastry, et al. (2006). "Regulation of cell adhesion by protein-tyrosine phosphatases. I. Cell-matrix adhesion." J Biol Chem **281**(23): 15593-6.

Byth, K. F., L. A. Conroy, et al. (1996). "CD45-null transgenic mice reveal a positive regulatory role for CD45 in early thymocyte development, in the selection of CD4+CD8+ thymocytes, and B cell maturation." J Exp Med **183**(4): 1707-18.

Cary, L. A., D. C. Han, et al. (1998). "Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration." J Cell Biol **140**(1): 211-21.

Cavallaro, U. and G. Christofori (2004). "Cell adhesion and signalling by cadherins and Ig-CAMs in cancer." Nat Rev Cancer **4**(2): 118-32.

Chiarugi, P. and P. Cirri (2003). "Redox regulation of protein tyrosine phosphatases during receptor tyrosine kinase signal transduction." Trends Biochem Sci **28**(9): 509-14.

Chiarugi, P., M. L. Taddei, et al. (2005). "Oxidation and tyrosine phosphorylation: synergistic or antagonistic cues in protein tyrosine phosphatase." Cell Mol Life Sci **62**(9): 931-6.

Chim, C. S., T. K. Fung, et al. (2004). "SOCS1 and SHP1 hypermethylation in multiple myeloma: implications for epigenetic activation of the Jak/STAT pathway." Blood **103**(12): 4630-5.

Cook, W. S. and R. H. Unger (2002). "Protein tyrosine phosphatase 1B: a potential leptin resistance factor of obesity." Dev Cell **2**(4): 385-7.

Dadke, S., A. Kusari, et al. (2001). "Phosphorylation and activation of protein tyrosine phosphatase (PTP) 1B by insulin receptor." Mol Cell Biochem **221**(1-2): 147-54.

Davies, H., G. R. Bignell, et al. (2002). "Mutations of the BRAF gene in human cancer." Nature **417**(6892): 949-54.

Dignass, A. U., K. Lynch-Devaney, et al. (1994). "Hepatocyte growth factor/scatter factor modulates intestinal epithelial cell proliferation and migration." Biochem Biophys Res Commun **202**(2): 701-9.

Dube, N., A. Bourdeau, et al. (2005). "Genetic ablation of protein tyrosine phosphatase 1B accelerates lymphomagenesis of p53-null mice through the regulation of B-cell development." Cancer Res **65**(21): 10088-95.

Elchebly, M., P. Payette, et al. (1999). "Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene." Science **283**(5407): 1544-8.

Elchebly, M., J. Wagner, et al. (1999). "Neuroendocrine dysplasia in mice lacking protein tyrosine phosphatase σ." Nat Genet **21**(3): 330-3.

Fachinger, G., U. Deutsch, et al. (1999). "Functional interaction of vascular endothelial-protein-tyrosine phosphatase with the angiopoietin receptor Tie-2." Oncogene **18**(43): 5948-53.

Freiss, G. and F. Vignon (2004). "Protein tyrosine phosphatases and breast cancer." Crit Rev Oncol Hematol **52**(1): 9-17.

Gaillard, C. and F. Strauss (1990). "Ethanol precipitation of DNA with linear polyacrylamide as carrier." Nucleic Acids Res **18**(2): 378.

Gallego, M. and D. M. Virshup (2005). "Protein serine/threonine phosphatases: life, death, and sleeping." Curr Opin Cell Biol **17**(2): 197-202.

Gee, C. E. and I. M. Mansuy (2005). "Protein phosphatases and their potential implications in neuroprotective processes." Cell Mol Life Sci **62**(10): 1120-30.

Glei, M. and B. L. Pool-Zobel (2006). "The main catechin of green tea, (-)-epigallocatechin-3-gallate (EGCG), reduces bleomycin-induced DNA damage in human leucocytes." Toxicol In Vitro **20**(3): 295-300.

Gosse, F., S. Guyot, et al. (2005). "Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis." Carcinogenesis **26**(7): 1291-5.

Graziani, G., G. D'Argenio, et al. (2005). "Apple polyphenol extracts prevent damage to human gastric epithelial cells *in vitro* and to rat gastric mucosa *in vivo*." Gut **54**(2): 193-200.

Hague, A., A. J. Butt, et al. (1996). "The role of butyrate in human colonic epithelial cells: an energy source or inducer of differentiation and apoptosis?" Proc Nutr Soc **55**(3): 937-43.

Hague, A. and C. Paraskeva (1995). "The short-chain fatty acid butyrate induces apoptosis in colorectal tumour cell lines." Eur J Cancer Prev **4**(5): 359-64.

Hague, A., B. Singh, et al. (1997). "Butyrate acts as a survival factor for colonic epithelial cells: further fuel for the *in vivo* versus *in vitro* debate." Gastroenterology **112**(3): 1036-40.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Harris, A. L. (2002). "Hypoxia-a key regulatory factor in tumour growth." Nat Rev Cancer **2**(1): 38-47.

Hassig, C. A., J. K. Tong, et al. (1997). "Fiber-derived butyrate and the prevention of colon cancer." Chem Biol **4**(11): 783-9.

Heldin, C. H. (1995). "Dimerization of cell surface receptors in signal transduction." Cell **80**(2): 213-23.

Holsinger, L. J., K. Ward, et al. (2002). "The transmembrane receptor protein tyrosine phosphatase DEP1 interacts with p120 (ctn)." Oncogene **21**(46): 7067-76.

Honda, H., J. Inazawa, et al. (1994). "Molecular cloning, characterization, and chromosomal localization of a novel protein-tyrosine phosphatase, HPTP η." Blood **84**(12): 4186-94.

Hooft van Huijsduijnen, R., S. Walchli, et al. (2002). "Protein tyrosine phosphatases as drug targets: PTP1B and beyond." Expert Opin Ther Targets **6**(6): 637-47.

Hunter, T. (2000). "Signaling--2000 and beyond." Cell **100**(1): 113-27.

Iervolino, A., R. Iuliano, et al. (2006). "The receptor-type protein tyrosine phosphatase J antagonizes the biochemical and biological effects of RET-derived oncoproteins." Cancer Res **66**(12): 6280-7.

Iuliano, R., I. Le Pera, et al. (2004). "The tyrosine phosphatase PTPRJ/DEP-1 genotype affects thyroid carcinogenesis." Oncogene **23**(52): 8432-8.

Iuliano, R., F. Trapasso, et al. (2003). "An Adenovirus Carrying the Rat Protein Tyrosine Phosphatase η Suppresses the Growth of Human Thyroid Carcinoma Cell Lines *in Vitro* and *in Vivo*." Cancer Res **63**(4): 882-886.

Jandt, E., K. Denner, et al. (2003). "The protein-tyrosine phosphatase DEP-1 modulates growth factor-stimulated cell migration and cell-matrix adhesion." Oncogene **22**(27): 4175-85.

Jiang, G., J. den Hertog, et al. (1999). "Dimerization inhibits the activity of receptor-like protein-tyrosine phosphatase-σ." Nature **401**(6753): 606-10.

Kaplan, K. B., J. R. Swedlow, et al. (1995). "c-Src enhances the spreading of src-/- fibroblasts on fibronectin by a kinase-independent mechanism." Genes Dev **9**(12): 1505-17.

Keane, M. M., G. A. Lowrey, et al. (1996). "The protein tyrosine phosphatase DEP-1 is induced during differentiation and inhibits growth of breast cancer cells." Cancer Res **56**(18): 4236-43.

Keilhack, H., F. S. David, et al. (2005). "Diverse biochemical properties of Shp2 mutants. Implications for disease phenotypes." J Biol Chem **280**(35): 30984-93.

Kellie, S., G. Craggs, et al. (2004). "The tyrosine phosphatase DEP-1 induces cytoskeletal rearrangements, aberrant cell-substratum interactions and a reduction in cell proliferation." J Cell Sci **117**(4): 609-618.

Kern, M., Z. Tjaden, et al. (2005). "Inhibitors of the epidermal growth factor receptor in apple juice extract." Mol Nutr Food Res **49**(4): 317-28.

Kitamura, S., S. Kondo, et al. (2000). "Met/HGF receptor modulates bcl-w expression and inhibits apoptosis in human colorectal cancers." Br J Cancer **83**(5): 668-73.

Klemke, R. L., J. Leng, et al. (1998). "CAS/Crk coupling serves as a "molecular switch" for induction of cell migration." J Cell Biol **140**(4): 961-72.

Kovalenko, M., K. Denner, et al. (2000). "Site-selective dephosphorylation of the platelet-derived growth factor β-receptor by the receptor-like protein-tyrosine phosphatase DEP-1." J Biol Chem **275**(21): 16219-26.

Lampugnani, M. G., A. Zanetti, et al. (2003). "Contact inhibition of VEGF-induced proliferation requires vascular endothelial cadherin, β-catenin, and the phosphatase DEP-1/CD148." J. Cell Biol. **161**(4): 793-804.

Larsen, M., M. L. Tremblay, et al. (2003). "Phosphatases in cell-matrix adhesion and migration." Nat Rev Mol Cell Biol **4**(9): 700-11.

Lee, S. R., K. S. Yang, et al. (2002). "Reversible inactivation of the tumor suppressor PTEN by H_2O_2." J Biol Chem **277**(23): 20336-42.

Lesueur, F., P. D. Pharoah, et al. (2005). "Allelic association of the human homologue of the mouse modifier *Ptprj* with breast cancer." Hum Mol Genet **14**(16): 2349-56.

Levi, F., C. Pasche, et al. (1999). "Food groups and colorectal cancer risk." Br J Cancer **79**(7-8): 1283-7.

Li, L. and J. E. Dixon (2000). "Form, function, and regulation of protein tyrosine phosphatases and their involvement in human diseases." Semin Immunol **12**(1): 75-84.

Lim, Y., I. Han, et al. (2004). "Phosphorylation of focal adhesion kinase at tyrosine 861 is crucial for Ras transformation of fibroblasts." J Biol Chem **279**(28): 29060-5.

Lin, J. and A. Weiss (2003). "The tyrosine phosphatase CD148 is excluded from the immunologic synapse and down-regulates prolonged T cell signaling." J Cell Biol **162**(4): 673-82.

Liu, R. H. and J. Sun (2003). "Antiproliferative activity of apples is not due to phenolic-induced hydrogen peroxide formation." J Agric Food Chem **51**(6): 1718-23.

Lu, W., K. Shen, et al. (2003). "Chemical dissection of the effects of tyrosine phosphorylation of SHP-2." Biochemistry **42**(18): 5461-8.

Lu, Y. R. and L. Y. Foo (1997). "Identification and quantification of major polyphenols in apple pomace." Food Chemistry **59**(2): 187-194.

Lu, Y. R. and L. Y. Foo (2000). "Antioxidant and radical scavenging activities of polyphenols from apple pomace." Food Chemistry **68**(1): 81-85.

Luo, L., G. Q. Shen, et al. (2006). "Loss of heterozygosity in human aberrant crypt foci (ACF), a putative precursor of colon cancer." Carcinogenesis **27**(6): 1153-9.

MacKeigan, J. P., L. O. Murphy, et al. (2005). "Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance." Nat Cell Biol **7**(6): 591-600.

Manning, G., D. B. Whyte, et al. (2002). "The protein kinase complement of the human genome." Science **298**(5600): 1912-34.

Massa, A., F. Barbieri, et al. (2004). "The Expression of the Phosphotyrosine Phosphatase DEP-1/PTPη Dictates the Responsivity of Glioma Cells to Somatostatin Inhibition of Cell Proliferation." J. Biol. Chem. **279**(28): 29004-29012.

Meng, K., A. Rodriguez-Pena, et al. (2000). "Pleiotrophin signals increased tyrosine phosphorylation of β-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase β/ζ." Proc Natl Acad Sci U S A **97**(6): 2603-8.

Meng, T. C., D. A. Buckley, et al. (2004). "Regulation of insulin signaling through reversible oxidation of the protein-tyrosine phosphatases TC45 and PTP1B." J Biol Chem **279**(36): 37716-25.

Meng, T. C., T. Fukada, et al. (2002). "Reversible oxidation and inactivation of protein tyrosine phosphatases *in vivo*." Mol Cell **9**(2): 387-99.

Mitra, S. K., D. A. Hanson, et al. (2005). "Focal adhesion kinase: in command and control of cell motility." Nat Rev Mol Cell Biol **6**(1): 56-68.

Moen, C. J., P. C. Groot, et al. (1996). "Fine mapping of colon tumor susceptibility (*Scc*) genes in the mouse, different from the genes known to be somatically mutated in colon cancer." Proc Natl Acad Sci U S A **93**(3): 1082-6.

Montagner, A., A. Yart, et al. (2005). "A novel role for Gab1 and SHP2 in epidermal growth factor-induced Ras activation." J Biol Chem **280**(7): 5350-60.

Motiwala, T., H. Kutay, et al. (2004). "Protein tyrosine phosphatase receptor-type O (PTPRO) exhibits characteristics of a candidate tumor suppressor in human lung cancer." Proc Natl Acad Sci U S A **101**(38): 13844-9.

Mutoh, M., M. Takahashi, et al. (2000). "Suppression by flavonoids of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells: structure-activity relationship." Jpn J Cancer Res **91**(7): 686-91.

Nam, H. J., F. Poy, et al. (1999). "Crystal structure of the tandem phosphatase domains of RPTP LAR." Cell **97**(4): 449-57.

Neel, B. G. and N. K. Tonks (1997). "Protein tyrosine phosphatases in signal transduction." Curr Opin Cell Biol **9**(2): 193-204.

Nishiya, N., W. B. Kiosses, et al. (2005). "An alpha4 integrin-paxillin-Arf-GAP complex restricts Rac activation to the leading edge of migrating cells." Nat Cell Biol **7**(4): 343-52.

O'Grady, P., T. C. Thai, et al. (1998). "The laminin-nidogen complex is a ligand for a specific splice isoform of the transmembrane protein tyrosine phosphatase LAR." J Cell Biol **141**(7): 1675-84.

Ohta, Y., J. H. Hartwig, et al. (2006). "FilGAP, a Rho- and ROCK-regulated GAP for Rac binds filamin A to control actin remodelling." Nat Cell Biol **8**(8): 803-14.

Ostman, A. and F. D. Bohmer (2001). "Regulation of receptor tyrosine kinase signaling by protein tyrosine phosphatases." Trends Cell Biol **11**(6): 258-66.

Ostman, A., C. Hellberg, et al. (2006). "Protein-tyrosine phosphatases and cancer." Nat Rev Cancer **6**(4): 307-20.

Ostman, A., Q. Yang, et al. (1994). "Expression of DEP-1, a receptor-like protein-tyrosine-phosphatase, is enhanced with increasing cell density." Proc Natl Acad Sci U S A **91**(21): 9680-4.

Palka, H. L., M. Park, et al. (2003). "Hepatocyte Growth Factor Receptor Tyrosine Kinase Met Is a Substrate of the Receptor Protein-tyrosine Phosphatase DEP-1." J. Biol. Chem. **278**(8): 5728-5735.

Parr, C. and W. G. Jiang (2006). "Hepatocyte growth factor activation inhibitors (HAI-1 and HAI-2) regulate HGF-induced invasion of human breast cancer cells." Int J Cancer **119**(5): 1176-83.

Peles, E., M. Nativ, et al. (1995). "The carbonic anhydrase domain of receptor tyrosine phosphatase β is a functional ligand for the axonal cell recognition molecule contactin." Cell **82**(2): 251-60.

Pera, I. L., R. Iuliano, et al. (2005). "The rat tyrosine phosphatase η increases cell adhesion by activating c-Src through dephosphorylation of its inhibitory phosphotyrosine residue." Oncogene **24**(19): 3187-95.

Persson, C., U. Engstrom, et al. (2002). "Primary sequence determinants responsible for site-selective dephosphorylation of the PDGF β-receptor by the receptor-like protein tyrosine phosphatase DEP-1." FEBS Lett **517**(1-3): 27-31.

Pixley, F. J., P. S. Lee, et al. (2001). "Protein tyrosine phosphatase ϕ regulates paxillin tyrosine phosphorylation and mediates colony-stimulating factor 1-induced morphological changes in macrophages." Mol Cell Biol **21**(5): 1795-809.

Podsypanina, K., L. H. Ellenson, et al. (1999). "Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems." Proc Natl Acad Sci U S A **96**(4): 1563-8.

Pool-Zobel, B., S. Veeriah, et al. (2005). "Modulation of xenobiotic metabolising enzymes by anticarcinogens-focus on glutathione S-transferases and their role as targets of dietary chemoprevention in colorectal carcinogenesis." Mutat Res **591**(1-2): 74-92.

Pool-Zobel, B. L., V. Selvaraju, et al. (2005). "Butyrate may enhance toxicological defence in primary, adenoma and tumor human colon cells by favourably modulating expression of glutathione S-transferases genes, an approach in nutrigenomics." Carcinogenesis **26**(6): 1064-76.

Rayapureddi, J. P., C. Kattamuri, et al. (2003). "Eyes absent represents a class of protein tyrosine phosphatases." Nature **426**(6964): 295-8.

Reed, J. C. (1999). "Dysregulation of apoptosis in cancer." J Clin Oncol **17**(9): 2941-53.

Ren, J. M., P. M. Li, et al. (1998). "Transgenic mice deficient in the LAR protein-tyrosine phosphatase exhibit profound defects in glucose homeostasis." Diabetes **47**(3): 493-7.

Richter, M., D. Jurek, et al. (2002). "Cells obtained from colorectal microadenomas mirror early premalignant growth patterns in vitro." Eur J Cancer **38**(14): 1937-45.

Ross, J. A. and C. M. Kasum (2002). "Dietary flavonoids: bioavailability, metabolic effects, and safety." Annu Rev Nutr **22**: 19-34.

Ruivenkamp, C., M. Hermsen, et al. (2003). "LOH of *PTPRJ* occurs early in colorectal cancer and is associated with chromosomal loss of 18q12-21." Oncogene **22**(22): 3472-4.

Ruivenkamp, C. A., T. van Wezel, et al. (2002). "*Ptprj* is a candidate for the mouse colon-cancer susceptibility locus *Scc1* and is frequently deleted in human cancers." Nat Genet **31**(3): 295-300.

Rupnarain, C., Z. Dlamini, et al. (2004). "Colon cancer: genomics and apoptotic events." Biol Chem **385**(6): 449-64.

Saha, S., A. Bardelli, et al. (2001). "A phosphatase associated with metastasis of colorectal cancer." Science **294**(5545): 1343-6.

Sallee, J. L., E. S. Wittchen, et al. (2006). "Regulation of cell adhesion by protein-tyrosine phosphatases: II. Cell-cell adhesion." J Biol Chem **281**(24): 16189-92.

Salmeen, A., J. N. Andersen, et al. (2003). "Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate." Nature **423**(6941): 769-73.

Salucci, M., L. A. Stivala, et al. (2002). "Flavonoids uptake and their effect on cell cycle of human colon adenocarcinoma cells (Caco2)." Br J Cancer **86**(10): 1645-51.

Sap, J., Y. P. Jiang, et al. (1994). "Receptor tyrosine phosphatase R-PTP-kappa mediates homophilic binding." Mol Cell Biol **14**(1): 1-9.

Schaapveld, R. Q., J. T. Schepens, et al. (1997). "Impaired mammary gland development and function in mice lacking LAR receptor-like tyrosine phosphatase activity." Dev Biol **188**(1): 134-46.

Schaefer, S., M. Baum, et al. (2006). "Polyphenolic apple juice extracts and their major constituents reduce oxidative damage in human colon cell lines." Mol Nutr Food Res **50**(1): 24-33.

Schaefer, S., M. Baum, et al. (2006). "Modulation of oxidative cell damage by reconstituted mixtures of phenolic apple juice extracts in human colon cell lines." Mol Nutr Food Res **50**(4-5): 413-7.

Schaller, M. D. (2004). "FAK and paxillin: regulators of N-cadherin adhesion and inhibitors of cell migration?" J Cell Biol **166**(2): 157-9.

Schaller, M. D. and J. T. Parsons (1995). "pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk." Mol Cell Biol **15**(5): 2635-45.

Semenza, G. L. (2003). "Targeting HIF-1 for cancer therapy." Nat Rev Cancer **3**(10): 721-32.

Serra-Pages, C., N. L. Kedersha, et al. (1995). "The LAR transmembrane protein tyrosine phosphatase and a coiled-coil LAR-interacting protein co-localize at focal adhesions." Embo J **14**(12): 2827-38.

Shimizu, M., A. Deguchi, et al. (2005). "EGCG inhibits activation of HER3 and expression of cyclooxygenase-2 in human colon cancer cells." J Exp Ther Oncol **5**(1): 69-78.

Shimizu, M., A. Deguchi, et al. (2005). "(-)-Epigallocatechin gallate and polyphenon E inhibit growth and activation of the epidermal growth factor receptor and human epidermal growth factor receptor-2 signaling pathways in human colon cancer cells." Clin Cancer Res **11**(7): 2735-46.

Soldani, C. and A. I. Scovassi (2002). "Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update." Apoptosis **7**(4): 321-8.

Song, G., G. Ouyang, et al. (2005). "The activation of Akt/PKB signaling pathway and cell survival." J Cell Mol Med **9**(1): 59-71.

Sorby, M., J. Sandstrom, et al. (2001). "An extracellular ligand increases the specific activity of the receptor-like protein tyrosine phosphatase DEP-1." Oncogene **20**(37): 5219-24.

Sulis, M. L. and R. Parsons (2003). "PTEN: from pathology to biology." Trends Cell Biol **13**(9): 478-83.

Takahashi, T., K. Takahashi, et al. (2006). "A monoclonal antibody against CD148, a receptor-like tyrosine phosphatase, inhibits endothelial cell growth and angiogenesis." Blood.

Takahashi, T., K. Takahashi, et al. (2003). A Mutant Receptor Tyrosine Phosphatase, CD148, Causes Defects in Vascular Development. Mol. Cell. Biol. **23:** 1817-1831.

Tao, J., C. C. Malbon, et al. (2001). "Insulin stimulates tyrosine phosphorylation and inactivation of protein-tyrosine phosphatase 1B *in vivo*." J Biol Chem **276**(31): 29520-5.

Tapiero, H., K. D. Tew, et al. (2002). "Polyphenols: do they play a role in the prevention of human pathologies?" Biomed Pharmacother **56**(4): 200-7.

Tartaglia, M., E. L. Mehler, et al. (2001). "Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome." Nat Genet **29**(4): 465-8.

Tebbutt, N. C., E. Cattell, et al. (2002). "Systemic treatment of colorectal cancer." Eur J Cancer **38**(7): 1000-15.

Tenev, T., S. A. Bohmer, et al. (2000). "Perinuclear localization of the protein-tyrosine phosphatase SHP-1 and inhibition of epidermal growth factor-stimulated STAT1/3 activation in A431 cells." Eur J Cell Biol **79**(4): 261-71.

Terry, P., E. Giovannucci, et al. (2001). "Fruit, vegetables, dietary fiber, and risk of colorectal cancer." J Natl Cancer Inst **93**(7): 525-33.

Tischer, C. and P. I. Bastiens (2003). "Lateral phosphorylation propagation: an aspect of feedback signalling?" Nat Rev Mol Cell Biol **4**(12): 971-4.

Tonks, N. K. (2005). "Redox redux: revisiting PTPs and the control of cell signaling." Cell **121**(5): 667-70.

Tonks, N. K., C. D. Diltz, et al. (1988). "Purification of the major protein-tyrosine-phosphatases of human placenta." J Biol Chem **263**(14): 6722-30.

Tonks, N. K. and B. G. Neel (2001). Combinatorial control of the specificity of protein tyrosine phosphatases. Curr Opin Cell Biol. **13:** 182-95.

Trapasso, F., A. Drusco, et al. (2006). "Genetic ablation of *Ptprj*, a mouse cancer susceptibility gene, results in normal growth and development and does not predispose to spontaneous tumorigenesis." DNA Cell Biol **25**(6): 376-82.

Trapasso, F., R. Iuliano, et al. (2000). "Rat protein tyrosine phosphatase η suppresses the neoplastic phenotype of retrovirally transformed thyroid cells through the stabilization of p27(Kip1)." Mol Cell Biol **20**(24): 9236-46.

Trapasso, F., S. Yendamuri, et al. (2004). "Restoration of receptor-type protein tyrosine phosphatase η function inhibits human pancreatic carcinoma cell growth *in vitro* and *in vivo*." Carcinogenesis: bgh224.

Tsubouchi, A., J. Sakakura, et al. (2002). "Localized suppression of RhoA activity by Tyr31/118-phosphorylated paxillin in cell adhesion and migration." J Cell Biol **159**(4): 673-83.

Turner, C. E. (2000). "Paxillin and focal adhesion signalling." Nat Cell Biol **2**(12): E231-6.

Uetani, N., K. Kato, et al. (2000). "Impaired learning with enhanced hippocampal long-term potentiation in PTPδ-deficient mice." Embo J **19**(12): 2775-85.

van Huijsduijnen, R. H., A. Bombrun, et al. (2002). "Selecting protein tyrosine phosphatases as drug targets." Drug Discov Today **7**(19): 1013-9.

Van Vactor, D., A. M. O'Reilly, et al. (1998). "Genetic analysis of protein tyrosine phosphatases." Curr Opin Genet Dev **8**(1): 112-26.

Veeriah, S., T. Kautenburger, et al. (2006). "Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics." Mol Carcinog **45**(3): 164-74.

Wang, Z., D. Shen, et al. (2004). "Mutational analysis of the tyrosine phosphatome in colorectal cancers." Science **304**(5674): 1164-6.

Webb, D. J., K. Donais, et al. (2004). "FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly." Nat Cell Biol **6**(2): 154-61.

Weinberg, R. A. (1991). "Tumor suppressor genes." Science **254**(5035): 1138-46.

Weinreb, O., S. Mandel, et al. (2003). "Gene and protein expression profiles of anti- and pro-apoptotic actions of dopamine, R-apomorphine, green tea polyphenol (-)-epigallocatechine-3-gallate, and melatonin." Ann N Y Acad Sci **993**: 351-61; discussion 387-93.

Weiss, A. and J. Schlessinger (1998). "Switching signals on or off by receptor dimerization." Cell **94**(3): 277-80.

Wells, A. and J. R. Grandis (2003). "Phospholipase C-γ1 in tumor progression." Clin Exp Metastasis **20**(4): 285-90.

Wenzel, U., S. Kuntz, et al. (2000). "Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells." Cancer Res **60**(14): 3823-31.

Wishart, M. J. and J. E. Dixon (2002). "PTEN and myotubularin phosphatases: from 3-phosphoinositide dephosphorylation to disease." Trends Cell Biol **12**(12): 579-85.

Witte, J. S., M. P. Longnecker, et al. (1996). "Relation of vegetable, fruit, and grain consumption to colorectal adenomatous polyps." Am J Epidemiol **144**(11): 1015-25.

Wolter, F., A. Clausnitzer, et al. (2002). "Piceatannol, a natural analog of resveratrol, inhibits progression through the S phase of the cell cycle in colorectal cancer cell lines." J Nutr **132**(2): 298-302.

Yano, H., Y. Mazaki, et al. (2004). "Roles played by a subset of integrin signaling molecules in cadherin-based cell-cell adhesion." J Cell Biol **166**(2): 283-95.

Yano, H., H. Uchida, et al. (2000). "Paxillin α and Crk-associated substrate exert opposing effects on cell migration and contact inhibition of growth through tyrosine phosphorylation." Proc Natl Acad Sci U S A **97**(16): 9076-81.

Yeatman, T. J. (2004). "A renaissance for SRC." Nat Rev Cancer **4**(6): 470-80.

Zaidel-Bar, R., C. Ballestrem, et al. (2003). "Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells." J Cell Sci **116**(Pt 22): 4605-13.

Zhang, S. Q., W. Yang, et al. (2004). "Shp2 regulates SRC family kinase activity and Ras/Erk activation by controlling Csk recruitment." Mol Cell **13**(3): 341-55.

Zheng, X. M., Y. Wang, et al. (1992). "Cell transformation and activation of pp60c-src by overexpression of a protein tyrosine phosphatase." Nature **359**(6393): 336-9.

6.2 Abbreviations

aa	Aminoacids
ACF	Aberrant Crypt Foci
APC	Adenomatous Polyposis Coli
AE	Apple polyphenol Extract
AJ	Apple juice
aPK	Atypical protein kinase
Asp	Aspartate
Atc	Anhydrotetracycline
But	Butyrate
Cdk	Cyclin Dependent Kinase
COX	Cyclo-oxygenase
CS (C1239S)	Cysteine – Serine mutant
Cy 2	Cyanine
Cy 3	Indocarbocyanine
Cys	Cysteine
DA	Aspartate – Alanine mutant
DAPI	4',6-Diamidino-2-phenylindole
DEP-1	Density Enhanced Phosphatase-1
DMH	1,2 - dimethyl hydrazine
DTAF	Dichlorotriazinylaminofluorescein
ECM	Extracellular matrix
EGCG	Epigallocatechin-3-gallate
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
Erk	Extracellular regulated kinase
ePK	Eukaryotic protein kinase
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum

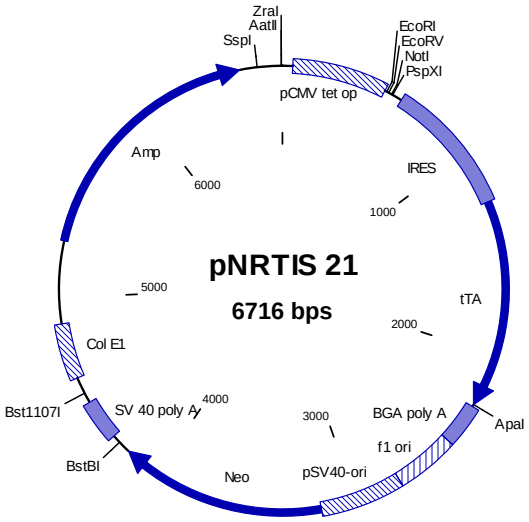
Fig.	Figure
FITC	Fluorescein Isothiocyanate
GAB2	Grb2 Associated Protein
GAP	GTPase Activating Protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosinediphosphate
GEF	Guanine nucleotide Exchange Factor
Grb2	Growth Factor Receptor bound 2
GSH	Glutathione Sepharose
GST	Glutathione-S-Transferase
GT	Green Tea
GTP	Guanosine-triphosphate
h	Hours
HGF	Hepatocyte Growth Factor
HGFR	Hepatocyte Growth Factor Receptor
HIF-1α	Hypoxia Inducible Factor 1 alpha
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horse radish peroxidase
IGF-1R	Insulin-like Growth Factor -1- Receptor
IP	Immunoprecipitation
IR	Insulin Receptor
JAK	Janus Kinase
kDa	Kilodalton
KO	Knock out
LB	Lysis buffer
LM PTP	Low Molecular Weight Protein Tyrosine Phosphatase
LOH	Loss of Heterozygosity
LPA	Lysophosphatidic acid
MACS	Magnetic Assisted Cell Sorter

Met	Methionine
min	Minutes
MAPK	Mitogen Activated Protein Kinase
MEK	MAP / Erk kinase
MKP	Map Kinase Phosphatase
MLCK	Myosin Light Chain Kinase
MMP	Matrix Metalloproteinase
NFkB	Nuclear Factor of kappa B Cells
NRPTP	Non-receptor Protein-Tyrosine Phosphatase
nt	Non-targeting control
PAE	Porcine Aortic Endothelial Cells
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly ADP-ribose polymerase
PDGFR	Platelet Derived Growth Factor Receptor
Ph eq	Phloridzin equivalents
PI3K	Phosphoinositide 3 kinase
PLC	Phospholipase C
PTK	Protein tyrosine kinase
PTP	Protein Tyrosine Phosphatase
PTP 1B	Protein Tyrosine Phosphatase 1B
pTyr	Phospho-tyrosine
Raf	Ras Activated Factor
Ras	Rat Sarcoma
RASGAP	Ras GTPase Activating Protein
ROS	Reactive Oxygen Species
RPTP	Receptor like Protein-Tyrosine Phosphatase
RT	Room temperature
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
RTK	Receptor Tyrosine Kinase

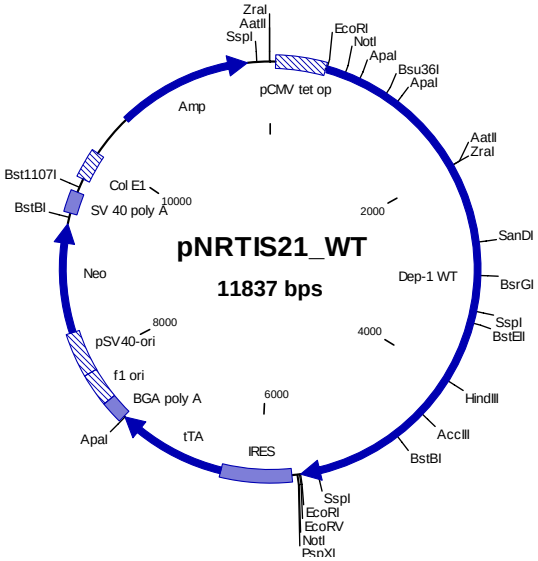
s	suppressed
S1P	Sphingosine-1-phosphate
Scc	Susceptibility to Colon Cancer
SCFA	Short chain fatty acids
SDF 1α	Stromal Cell Derived Factor 1 alpha
sec	seconds
Ser	Serine
SFKs	Src Family Kinases
siRNA	Short interfering RNA
SH	Src homology
shRNA	Short hairpin RNA
SOS	Son of Sevenless
STAT	Signal Transducers and Activators of Transcription
STS	Staurosporine
TCR	T cell receptor
Thr	Threonine
TRITC	Tetramethyl Rhodamine Isothiocyanate
tTA	Tetracycline-repressed Transactivator
Tyr	Tyrosine
VEGF	Vascular Endothelial Growth Factor
WT	Wild type

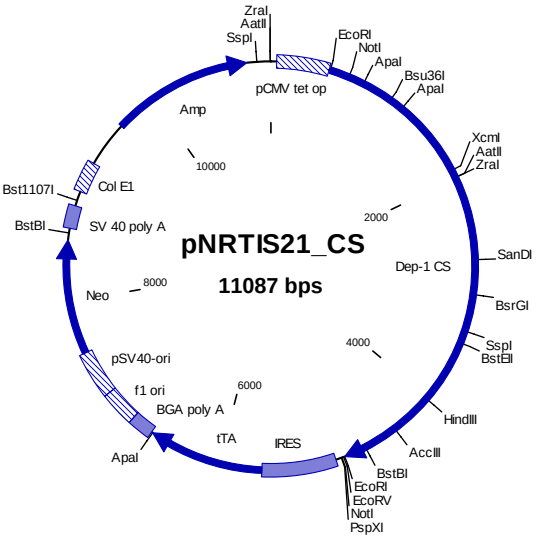
6.3. Plasmid Maps and Vector Constructs

Map of the inducible pNRTIS 21 vector

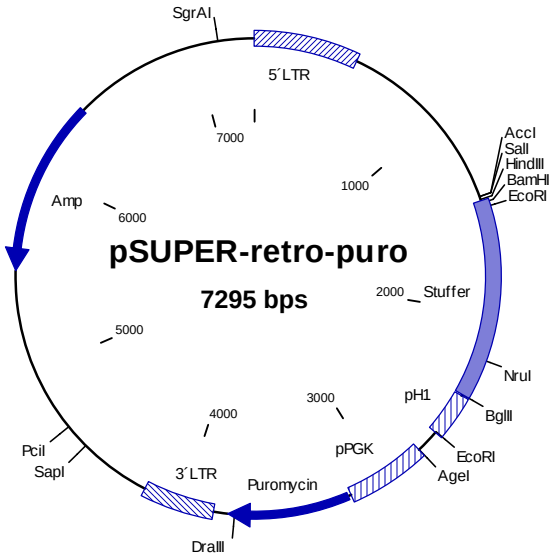


Maps of pNRTIS 21 vector harboring the DEP-1 WT and CS variants

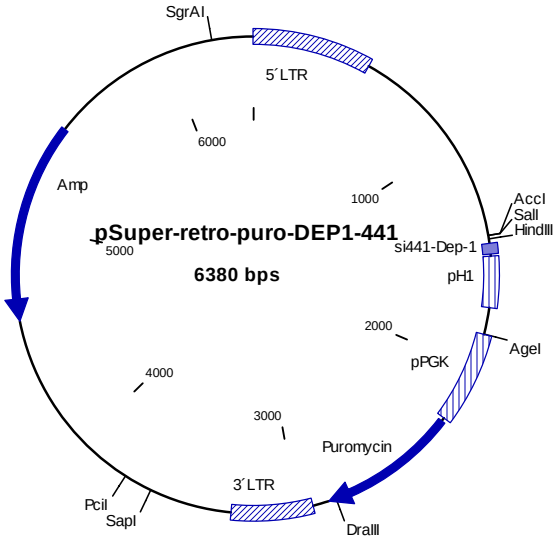




Map of pSUPER-retro-puro vector



Map of pSUPER-retro-puro vector containing the 64 nt shRNA construct (construct 441)



6.4. RTK Array

List of anti-RTK antibodies spotted on the membrane

Coordinate	Receptor Family	RTK/Control	Coordinate	Receptor Family	RTK/Control
A1, A2	Control (+)	PY-Control*	D1, D2	Tie	Tie-2
A23, A24	Control (+)	PY-Control*	D3, D4	NGF R	TrkA
B1, B2	EGF R	EGF R	D5, D6	NGF R	TrkB
B3, B4	EGF R	ErbB2	D7, D8	NGF R	TrkC
B5, B6	EGF R	ErbB3	D9, D10	VEGF R	VEGF R1
B7, B8	EGF R	ErbB4	D11, D12	VEGF R	VEGF R2
B9, B10	FGF R	FGF R1	D13, D14	VEGF R	VEGF R3
B11, B12	FGF R	FGF R2α	D15, D16	MuSK	MuSK
B13, B14	FGF R	FGF R3	D17, D18	Eph R	EphA1
B15, B16	FGF R	FGF R4	D19, D20	Eph R	EphA2
B17, B18	Insulin R	Insulin R	D21, D22	Eph R	EphA3
B19, B20	Insulin R	IGF-I R	D23, D24	Eph R	EphA4
B21, B22	Axl	Axl	E1, E2	Eph R	EphA6
B23, B24	Axl	Dtk	E3, E4	Eph R	EphA7
C1, C2	Axl	Mer	E5, E6	Eph R	EphB1
C3, C4	HGF R	HGF R	E7, E8	Eph R	EphB2
C5, C6	HGF R	MSP R	E9, E10	Eph R	EphB4
C7, C8	PDGF R	PDGF Rα	E11, E12	Eph R	EphB6
C9, C10	PDGF R	PDGF Rβ	E13, E14	Control (-)	Mouse IgG_1
C11, C12	PDGF R	SCF R	E15, E16	Control (-)	Mouse IgG_{2A}
C13, C14	PDGF R	Flt-3	E17, E18	Control (-)	Mouse IgG_{2B}
C15, C16	PDGF R	M-CSF R	E19, E20	Control (-)	Goat IgG
C17, C18	RET	c-Ret	E21, E22	Control (-)	PBS
C19, C20	ROR	ROR1	F1, F2	Control (+)	PY-Control*
C21, C22	ROR	ROR2	F23, F24	Control (+)	PY-Control*
C23, C24	Tie	Tie-1			

*Phospho-Tyrosine Positive Control

Transparency overlay used to the identify the corresponding RTK

6.5. Acknowledgement

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6.6. Lebenslauf

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Jena, den 11. September 2006

Kamal Kumar Balavenkatraman

6.7. Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmittel, Literatur und persönlichen Mitteilungen angefertigt habe.

Jena, den 11. September 2006

Kamal Kumar Balavenkatraman

6.8. Erklärung zur Bewerbung

Hiermit erkläre ich, dass ich mich mit der vorliegenden Arbeit an keiner anderen Hochschule um den akademischen Grad Doctor rerum naturalium beworben habe und dass ich weder früher noch gegenwärtig die Eröffnung eines Verfahrens zum Erwerb des oben genannten akademischen Grades an einer anderen Hochschule beantragt habe. Die Promotions-Ordnung der Biologisch-Pharmazeutischen-Fakultät der FSU Jena ist mir bekannt.

Jena, den 11. September 2006

Kamal Kumar Balavenkatraman

6.9. List of Posters and Publications

Some part of this work has already been presented or published at the following places.

Publications

- **KK Balavenkatraman**, E Jandt, K Friedrich, T Kautenburger, BL Pool-Zobel, A Östman and FD Böhmer.
  DEP-1 protein tyrosine phosphatase inhibits proliferation and migration of colon carcinoma cells and is upregulated by protective nutrients.
  Oncogene 2006, May 8 (Epub ahead of print-DOI:10.1038/sj.onc.1209647).

Posters presented

- **Kamal Kumar Balavenkatraman**, Enrico Jandt, Karlheinz Friedrich, Tanja Kautenburger, Beatrice Pool-Zobel, Frank-D.Böhmer
  Title: "Role Of The Tumor Suppressor DEP-1 In Colon Epithelial Cells"
  Presented at the FEBS-EMBO Advanced Lecture Course on Molecular Mechanisms in Signal Transduction and Cancer at Spetses, Greece. (Aug, 2005)

- **B Kamal Kumar**, E Jandt, K Friedrich, T Kautenburger, B Pool-Zobel, D Böhmer
  Title: "Expression Regulation of the Protein-Tyrosine Phosphatase DEP-1 in Colon Cancer Cell Lines"
  Presented at the FEBS Lecture Course on Cellular Signalling & 4th Dubrovnik Signaling Conference at Dubrovnik, Croatia. (May 2004)

- **Kamal Kumar B**, Enrico Jandt, Karlheinz Friedrich, Tanja Kautenburger, Beatrice Pool-Zobel, Frank D. Böhmer
  Title: "Expression of DEP-1 in colon carcinoma cell lines and the effect of nutrient components" – Won a special poster prize.
  Presented at the 7th Joint Meeting - Signal Transduction-Receptors, Mediators and Genes conducted by Signal Transduction Society at Weimar (Nov 2003).